THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

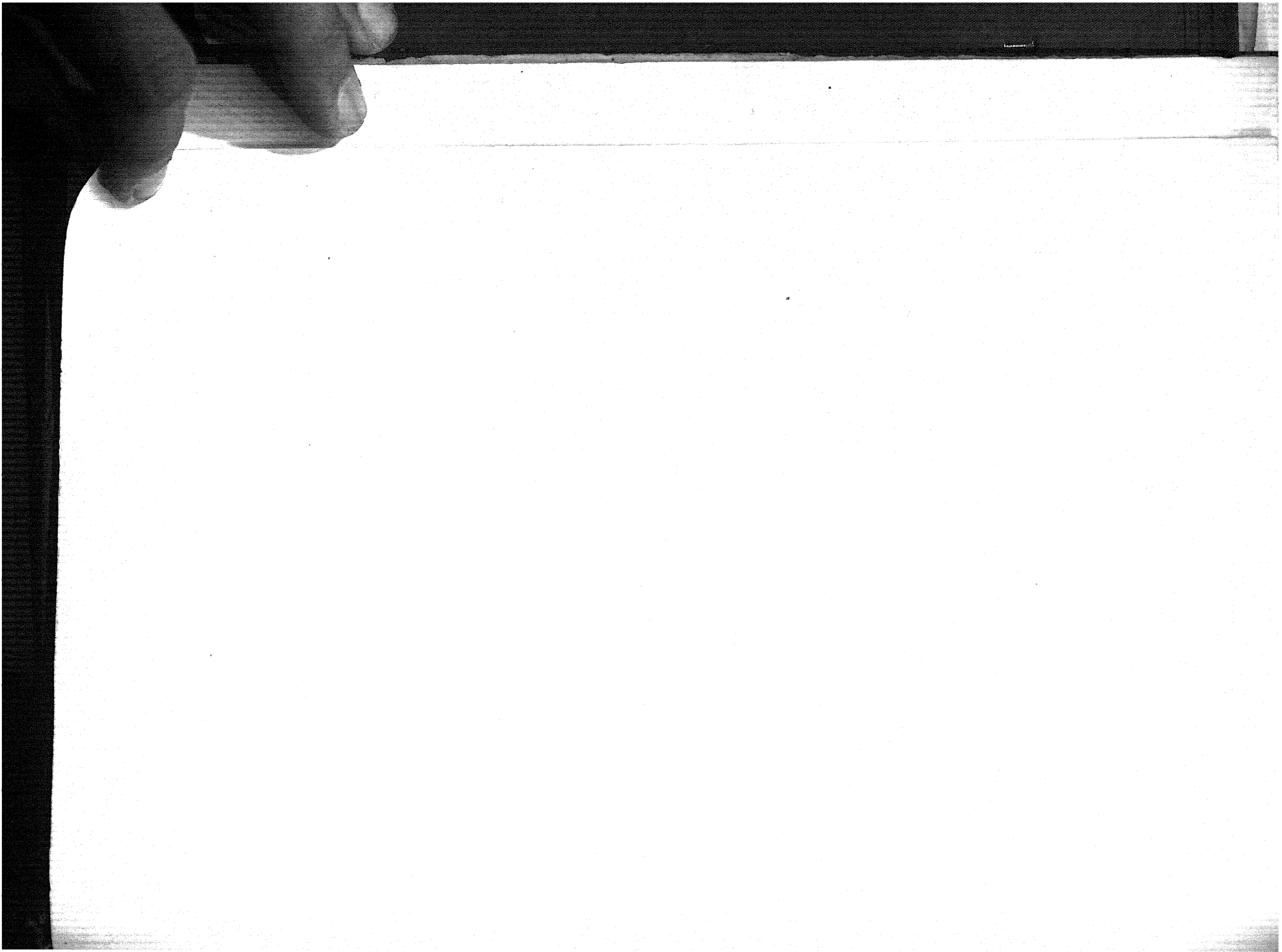
EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
SIR F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

VOLUME XXV 1931

PART II, pp. 973 to end

CAMBRIDGE
AT THE UNIVERSITY PRESS
1931



CVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

III. A METHOD FOR THE MEASUREMENT OF RESPIRATORY QUOTIENT IN SERUM.

By FRANK DICKENS[1] and FRANTIŠEK ŠIMER.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

(*Received May 10th, 1931.*)

In Part I of this series [Dickens and Šimer, 1930] the principles applicable to the measurement of the respiratory quotient of surviving tissues *in vitro* were summarised. It was concluded that the only existing principle by which the true R.Q. could be measured correctly, regardless of changes in acid-base equilibrium, was that in which the tissue is suspended in a CO_2-free medium, such as a Ringer solution buffered with phosphate, the gas-space containing pure CO_2-free oxygen. A reliable method involving such a principle was there described.

The method gives accurate results and is convenient and simple to use. Nevertheless, there are two obvious objections which may be brought against the conditions of the tissue in such a method; firstly, the low CO_2 tension, which approaches zero in the medium, and secondly, the fact that the whole of the buffering is due to phosphate, whilst in the body phosphate buffers are, at least quantitatively, much less important. Above all, the phosphate method cannot be applied if serum or other similar body-fluids are used as medium. There can be no doubt that at any rate in the case of certain delicate material, such as germinal and embryonic tissues, and probably for other tissues also, serum is a more suitable medium than a simple salt solution.

When an attempt is made to find a method which might be applied to the measurement of respiration and respiratory quotient in such a medium, very great practical difficulties arise. These are due to the circumstance remarked on by Warburg [1925] who wrote: "Up to now, no method exists for the estimation of the respiration CO_2, when fluids are employed whose bicarbonate content corresponds to that of serum, *i.e.* under conditions which are physiological for cells of the higher animals."

In the present paper, a method is described whereby the CO_2 formed in respiration by a piece of tissue suspended in such a medium may be directly measured, independently of any changes whatever in the bound CO_2, whether these changes arise from production or consumption of acids by the tissue, or

[1] A member of the scientific staff of the Medical Research Council.

whether they are due to retention of CO_2 by the buffer substances contained in the medium. The oxygen consumption of the same piece of tissue is given simultaneously by manometric readings, and hence the R.Q. is accurately determined. The method may therefore be used for determining the R.Q. and respiration, either with simple Ringer solution containing bicarbonate, or with serum or other similar fluid in equilibrium with the physiological tension of CO_2. A great advantage of the method is that exactly the same technique and calculation may be used with equal accuracy either for Ringer solution or serum, and no corrections, empirical or otherwise, are needed for the "retention" in the latter medium.

Principle. The method is a manometric one. A thin slice of the tissue is suspended in serum or Ringer solution in a vessel of special design, filled with a mixture of oxygen with the required percentage of CO_2. The vessel and contents, connected with a simple Barcroft-Warburg manometer, are shaken in a thermostat as in Warburg's method [1925]. If we denote the total pressure-change read on the scale of the manometer during the experimental period by h, this pressure, which may be either positive or negative, is made up of the following:

(1) h_{O_2} = pressure-change caused by disappearance of oxygen consumed in respiration;

and (2) h_{CO_2} = pressure-change due to respiratory CO_2;

and (3) h_b = pressure-change due to alteration in the amount of combined CO_2 during the experiment.

These magnitudes are related to the observed pressure-change h, by the following equation:

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

In this equation all pressures must be algebraically reckoned, h_{O_2} being always negative, h_{CO_2} positive, and h_b either positive or negative according to whether the total amount of chemically combined CO_2 is diminished or increased.

The magnitude of h_b is determined by liberation of the whole of the bound CO_2 present at the beginning and end of the experiment by acidification with excess of strong acid.

In order to determine h_{CO_2} a new principle has been introduced. A direct determination is made of the change during the experimental period in the total amount of CO_2, bound and free, present in the vessel. This quantity is the amount of CO_2 respired during the experiment and is denoted by x_{CO_2}. It is important to emphasise that by the determination of this magnitude, three of the four quantities in equation (1) are known, namely, h, h_{CO_2} and h_b. Hence the value of h_{O_2} is determined, and therefore the amount of respired oxygen (x_{O_2}) is also known.

Both x_{O_2} and x_{CO_2} being known, the R.Q. $= -x_{CO_2}/x_{O_2}$.

It will be noted that the above principle is quite general for any medium.

METHOD.

Apparatus and solutions. The special vessels[1] (Fig. 1) used to contain the tissue and solutions are attached by means of the ground-joint to Barcroft manometers. The vessels are conical in shape, with as nearly as possible equal volumes of about 20 cc. They contain an inner separate conical part (*A*) which receives the piece of tissue and the medium (1·5 cc. serum or Ringer solution).

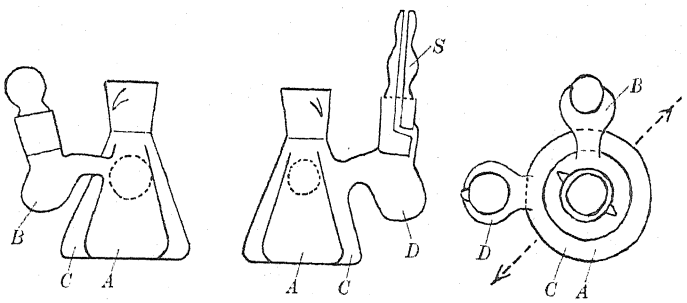


Fig. 1. The dotted line in the plan shows direction of shaking.

Communicating with compartment *A* by a fused-through join is the bulb *B*, containing excess of acid (0·1 cc. 3*N* HCl) which may at any desired moment be tipped into *A* by inclining the vessel. In this way, the respiration is stopped and the total bound CO_2 is liberated.

The outer annular portion *C*, and the bulb *D* communicating with it, serve for the absorption of the whole of the CO_2 contained in the vessel. For this purpose, use has been made of a principle of gas-analysis described by Krebs [1930]. The part *C* contains 2 cc. of a solution of $KMnO_4$ (*M*/5 with *M*/500 H_2SO_4), whilst bulb *D* has 0·2 cc. 30 % NaI, the latter solution being acidified to *N*/500 with H_2SO_4 immediately before use. When the contents of the bulb *D* are tipped into the part *C*, the iodide reacts with the $KMnO_4$ and a strongly alkaline solution results. The free CO_2 is thus rapidly and quantitatively absorbed.

The stopper *S* is of the type used by Warburg and Kubowitz [1929] for the determination of oxygen in gas-mixtures. It is closed by turning the stopper under the water of the bath, and permits the streaming of gas through the vessel whilst the latter is being shaken in the thermostat. Its use is essential for the accuracy of the method. An ordinary glass stopper is used for the other bulb (*B*).

Manometric fluid. The usual Brodie fluid (sp. gr. 1·033) is unsuitable for these experiments since its use would necessitate inconveniently long manometers in order to measure the large pressure changes involved. According to the carbon dioxide tension in the vessel, one or other of two different fluids has been found satisfactory.

[1] The vessels were made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

(1) For CO_2 tensions of the order of that occurring in serum, Clerici solution as used by mineralogists (a mixture of thallium malonate and thallium formate of sp. gr. approximately 4) is used. Sodium tauroglycocholate (0·1 %) is dissolved in the solution before use and, after filtration, the sp. gr. of the mixture at the prevailing temperature of the laboratory is accurately determined.

(2) In some experiments where a lower CO_2 tension than the physiological is employed, a solution of lead perchlorate, sp. gr. 2·07 [Krebs, 1930], has been used instead of the above solution.

These solutions must be protected from accidental dilution by droplets of water by means of loosely-fitting glass caps on the open ends of the manometers and by cotton-wool plugs inserted loosely in the tubes supplying the moist gas stream to the vessels. If new rubber tubing is used for the reservoirs of the manometers, it should be artificially aged by soaking in Clerici solution before use to remove traces of an impurity which otherwise slowly reacts with the solution, forming a dark-coloured deposit on the glass.

Supply of gas mixture. The gas mixture stored under pressure in the usual type of commercial gas-cylinder is first passed through a simple T-tube safety device, consisting of a vertical tube immersed to a suitable depth in water, which controls the maximum pressure of gas in the manometers. It then passes through a little water contained in a bubbler immersed in the bath, and the moist gas is distributed to the manometers through a six-way tube. Before entering the manometers the gas passes through the small tubes containing the cotton-wool plugs already mentioned; these tubes are attached by flexible rubber connections to the gas supply in order to allow vigorous shaking during the passage of the gas.

Procedure. For each determination, two vessels with tissue are necessary, or more if duplicate estimations are to be made. The solutions are first pipetted into the vessels as described above, serum having first been approximately saturated with the gas-mixture used in the experiment. Special care is necessary to measure exactly equal volumes of the serum or $NaHCO_3$-Ringer into all vessels. The fresh tissue is then cut with the razor in the usual way, with care to cause minimal damage to the tissue and to secure slices sufficiently thin to permit complete diffusion of metabolites [Warburg, 1925]. Before being transferred to the vessels, the slices are first suspended in serum or $NaHCO_3$-Ringer solution and a stream of 5 % carbon dioxide is passed briskly through for some 5 minutes, in order to arrive at a uniform preliminary condition in the various slices. They are then weighed in the moist state on a torsion balance, the weight for each vessel being equal, and the amount sufficient to give a respiration of some 100 $mm.^3$ per hour. After weighing each piece of tissue it is quickly rinsed in a $NaHCO_3$-free salt solution. This stage is not essential, but tends to lead to more consistent figures for the $NaHCO_3$-introduced with the tissue into the vessel. The tissue is then placed in the central compartment of the vessels, into the serum or $NaHCO_3$-Ringer. The vessels are attached to

their respective manometers and are together put into the bath with their stoppers closed. A third vessel without tissue and containing only the $KMnO_4$ and NaI is used for the analysis of the gas mixtures, and as usual the bath also contains a thermobarometric control vessel. The gas stream (see above) is now connected to the upper tubes of all manometers and a positive pressure of gas is set up in the vessels. The stoppers S of the vessels are now opened under the water, without removing the vessels from the bath, until a vigorous gas-stream is seen to bubble through the water of the bath. At the same time it is necessary to make sure that the vessels are firmly fixed to their manometers by means of their ground-joints. The shaking apparatus is now set in motion and the time noted. A period of 10 minutes, during which a steady stream of gas passes and the shaking is maintained at the rate of 90 swings per minute, suffices for thermal and gaseous equilibrium to be established. This rate of shaking must be used throughout the experiment. During the preliminary period the levels of the manometric fluid are roughly adjusted to a suitable height. The gas-stream is now stopped by turning the stoppers S without removing the vessels from the bath, the shaking being momentarily interrupted for this purpose. The upper taps of the manometers are closed, the tubing used for the gas-supply disconnected, and the final adjustment of level in the manometers is made. Shaking is recommenced, and the first reading of all vessels is taken 15 minutes from the time of first putting into the bath. Immediately after taking this reading, the acid from bulb B is at once tipped into the control vessels, which are momentarily removed from the bath for this purpose. The positive pressure caused by this acidification is read off and is denoted by h_{B_0} in the Protocol at the end of this paper, in which vessels Nos. 7 and 11 are the controls on the preformed CO_2, and Nos. 9 and 10 are the duplicate experimental vessels used for a double determination of R.Q., whilst No. 12 is solely for analysis of the gas-mixture from the cylinder.

During the experimental period of about 3 hours, the shaking of manometers is interrupted at intervals of half hour in order to follow the change of pressure in Nos. 9 and 10, the difference between the initial pressure and final pressures at the end of the 3 hour period, corrected of course for any small changes shown by the thermobarometer vessel, gives the value of h. Immediately after reading this quantity, the acid is tipped into the experimental vessels, causing a positive pressure (h_{B_1}) which is read off. After adding the acid, about 10 minutes is sufficient for the complete evolution of CO_2.

The whole of the CO_2 in the vessels is now in the free state, and is determined in all vessels, including No. 12, used for analysis of the gas-mixture, by tipping the iodide into the $KMnO_4$.

The absorption of CO_2 is complete in some 20 minutes, and the resulting negative pressures, H_0, H_1, and H_{gas} are then read off on the respective manometers of the control, experimental, and gas-analysis vessels.

Calculation. The basis of the calculation is the relationship given in equation (1):

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

Since the volumes of gas corresponding to these heights are merely the products of these heights in mm. and the appropriate vessel-constants, equation (1) may be re-written in the form:

$$h = \frac{x_{O_2}}{k_{O_2}} + \frac{x_{CO_2} + x_b}{k_{CO_2}} \qquad \text{.....(2).}$$

Vessel constants. For a manometric fluid of specific gravity S, the calculation of the vessel-constants for oxygen and carbon dioxide is as follows:

$$k = \frac{V_G \times (273/T) + V_F . \alpha}{760 \times (13{\cdot}60/S)},$$

where $V_G + V_F$ = total volume in mm.^{3} of vessel and capillary of manometer to the zero mark, V_F = volume of liquid placed in manometer vessel and α = Bunsen solubility coefficient of oxygen or CO_2 respectively. S must be accurately determined for each batch of manometer fluid prepared.

In the following calculations, quantities determined in the experimental vessel I (at time t_1), and at time t_0 in the control (vessel 0) are denoted by the suffixes 1 and 0 and the vessel-constants for carbon dioxide by k_1 and k_0 respectively, whilst k_{O_2} is the constant for oxygen relating to the experimental vessel.

Determination of x_{CO_2}. The total amount of CO_2 in the vessel is made up of the following: (1) CO_2 introduced as gas at the time of filling the vessel, (2) bound CO_2 introduced with the tissue and serum or Ringer bicarbonate, (3) CO_2 formed in respiration. Each of these will now be considered.

(1) Since all the vessels are filled with the same gas-mixture under the same pressure, all contain the same percentage of CO_2 at the time of closing the taps. By absorption of this amount of CO_2 the negative pressure H_{gas} is set up, which is related to the percentage of CO_2 in the gas-mixture used by the following equation [Krebs, 1930]:

$$\%\ CO_2 = \frac{H_{gas} \times 100}{P_o - p_w} \qquad \text{.....(3),}$$

where P_o = total pressure inside the vessel at the beginning of the experiment, and p_w = pressure of saturated vapour above the solutions in the vessel, all pressures being reckoned as usual in heights of manometer fluid.

This equation shows that H_{gas} is independent of the size of vessel or amount of fluid contained in it, and for a certain gas-mixture is constant if the total pressure in the vessels is the same. In any particular experiment this is easily realised by making the initial levels of manometer fluid in the two limbs approximately equal. The actual amount of CO_2 is of course dependent on the size of the vessel and amount of fluid, and is given by $k_{CO_2} . H_{gas}$.

(2) If the quantities of solution and tissue are the same in experimental and control vessels, the amount of bicarbonate introduced is the same in both vessels and is affected to the same extent in both vessels up to the time t_0.

(3) The same applies to respiration, which up to the time t_0 is the same in both vessels. In any case, this term is very small, since it concerns only the amount of respiratory CO_2 produced in the interval between stopping the gas-stream and t_0, which is in general only 5 minutes or less.

The volume of CO_2 produced between the times t_0 and t_1 in the experimental vessel, *i.e.* respiratory $CO_2 = x_{CO_2}$, is given by the difference in initial and final contents of total CO_2 in the vessel. If the vessels were of exactly the same size, x_{CO_2} would be simply the difference in the amounts of CO_2 present in the control vessel at t_0 and the experimental vessel at t_1. Since in practice we are dealing with vessels of slightly different volume, this must be considered in the calculation, which is carried out by either of the two principles described below.

First principle.

The quantities actually measured are H_1 and H_0, from which we may at once subtract H_{gas}, since this is the same in both vessels. Hence, apart from the amount of free CO_2 introduced in the gas-mixture, the amounts of CO_2 at time t_0 and t_1 are:

$$(H_0 - H_{gas})\, k_0 \text{ and } (H_1 - H_{gas})\, k_1$$

respectively. The difference between these two amounts is the respiratory CO_2 between time t_0 and t_1, hence:

$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - (H_0 - H_{gas})\, k_0 \qquad \text{.....(4).}$$

Determination of x_{O_2}. From equation (2):

$$x_{O_2} = \left(h - \frac{x_{CO_2} + x_b}{k_1}\right) k_{O_2} \qquad \text{.....(5).}$$

In this equation, x_b is the change in bound CO_2 during the experimental time $t_1 - t_0$. If B_0 = initial amount of bound CO_2 at the time t_0, and B_1 = bound CO_2 at time t_1 in the experimental vessel, then:

$$x_b = B_0 - B_1 = h_{B_0} k_0 - h_{B_1} k_1 \qquad \text{.....(6).}$$

The values x_{CO_2} and x_b and h in equation (5) being known, x_{O_2} is thus determined, and R.Q. $= -x_{CO_2}/x_{O_2}$ can at once be calculated.

Second principle.

By substituting the observed pressure changes from equations (4) and (6) in equation (5), this may also be written in the form:

$$h_{O_2} = h - H_1 + h_{B_1} + H_{gas}\{1 - (k_0/k_1)\} + (H_0 - h_{B_0})(k_0/k_1) \quad \text{...(7).}$$

Hence if $k_0 = k_1$, $h_{CO_2} = H_1 - H_0$ (from equation (4)) .....(4*a*),

and $h_{O_2} = h - H_1 + H_0 + h_{B_1} - h_{B_0}$ (from equation (7)) .....(8).

If the volumes of the two vessels are nearly the same, this condition can be achieved in practice, by varying the volume of liquid in these two vessels so that k_{CO_2} is the same for both. This modification makes the separate determination of H_{gas} unnecessary, provided as before that the quantities of tissue and serum taken are equal in the two vessels.

Notes.

(1) *Time of saturation with gas-mixture.* The time allowed is sufficient to attain complete filling of the vessel with the gas-mixture, and in control experiments the analysis was unchanged by prolonging the passage of gas for a greater time.

(2) *Control experiments with $NaHCO_3$ solutions.* In the absence of tissue in the vessel, x_{CO_2} is zero and $H - H_{gas} = h_B$, where h_B is the pressure change caused by the liberation of CO_2 from the bicarbonate taken. In numerous control experiments h_B and B $(= h_B.k_{CO_2})$ have been determined (*a*) by acidification and (*b*) from measurements of H and H_{gas} with an accuracy governed only by the accuracy of the manometer readings, *i.e.* $\pm$ 0·5 mm. manometer fluid.

(3) *Blank correction for tipping of NaI.* It should be mentioned that in determining the actual percentage of CO_2 in the vessel, it is necessary to apply the small correction of a few mm. mentioned by Krebs, due to the slight change of pressure which accompanies the mixing of the NaI and $KMnO_4$ and having no relation to the absorption of CO_2. In the method described, where the absolute percentage of CO_2 is not required, this correction is unnecessary, since it cancels out, as is clear from equations (4) and (8).

(4) *Magnitude of respiration and gaseous diffusion.* The fundamental principle underlying manometric measurements is the existence of a virtually complete equilibrium between the liquid and gaseous phases. The quantities of tissue and rate of shaking referred to above are suitably chosen to ensure this, and should be adhered to.

(5) *Correction for different weights of tissue.* With a little practice it is usually possible to weigh sufficiently nearly equal pieces of wet tissue into each vessel to render special correction for this point unnecessary; particularly as the magnitude of the correction for tissue taken from the control vessel is always small compared with the actual measurement in the experimental vessel. In all cases, however, the dry weight is accurately determined by drying to constant weight at 110°, since it is required for calculation of Q values [Warburg, 1930] and also as a check on the equality of the wet weights taken.

Should there be an appreciable difference between the dry weights of tissue in the control and experimental vessels, the correction may be applied in the following way. In the first place, the total bicarbonate content of the volume of original medium used is determined in the usual way. Denoting

this by B_M mm.^{3} and the weights of tissue by m_1 (experimental vessel) and m_0 (control vessel) then

$$x_b = B_M + (B_0 - B_M)\, m_1/m_0 - B_1,$$
$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - B_M - (H_0 k_0 - H_{gas} k_0 - B_M)\, m_1/m_0.$$

The remainder of the calculation is as given above.

Notes on the determination of glycolysis or oxidative removal of lactic acid.

(1) *In bicarbonate-Ringer solution.* In solutions containing no other buffer besides bicarbonate, the change in bound CO_2, x_b, is exactly equal to the amount of acid formed or consumed by the tissue (x_G):

$$x_b = x_G.$$

x_G is positive if acid is formed, *i.e.* CO_2 liberated, and negative if acid is consumed (*i.e.* CO_2 bound). Both quantities are expressed in mm.^{3} according to Warburg [1930].

From x_G the value of aerobic glycolysis is calculated in the usual way:

$$Q_M^{O_2} = x_G/(m.t),$$

where m is the dry weight of the tissue in mg. and t the duration of the experiment in hours.

The method described may be applied with equal accuracy to the measurement of oxidative removal of lactic, pyruvic or other organic acids metabolised by tissues.

(2) *In serum.* In serum the decrease of the amount of bicarbonate, x_b, is smaller than the amount of fixed acid, x_G, formed by the tissue. The difference, $x_G - x_b = \Delta x$, is due to the presence of buffers other than bicarbonate, which (1) neutralise part of the acid formed in glycolysis, and (2) combine with a certain amount of CO_2 formed in respiration.

Δx must be determined experimentally. Two vessels, O and I, of the type described in this paper (see Fig. 1) are used for the determination. Vessel I is the same vessel as that used in the main experiment.

Exactly equal amounts of a solution of tartaric acid are pipetted into bulb B of both vessels and evaporated to dryness at 100° [Warburg, 1925]. The concentration of the acid and the amounts pipetted must be chosen so that the quantity in bulb B of both vessels is as nearly as possible equal to x_G ($> x_b$). Into bulb D of both vessels is now pipetted a solution of tartaric acid of such concentration and volume that the quantity of acid is exactly equal to the respiratory CO_2, x_{CO_2}, of the main experiment. This condition is easily fulfilled as x_{CO_2} is accurately known from the main experiment. The volumes of tartaric acid solution in B and D are checked by weighing the vessel before and after pipetting, and a correction is applied if necessary.

The central compartment (A, Fig. 1) contains 1·5 cc. of serum in vessel I and 1·5 cc. bicarbonate-Ringer in vessel O. Into the outer part C of both vessels bicarbonate-Ringer solution is pipetted, the volume measured being

such that the total volume of fluid V_F is in both equal to that in the main experiment (3·80 cc.).

Both vessels are now filled with the same gas mixture in the same way as in the main experiment. After equilibrium has been established a reading of pressure is taken and in both vessels the acid in D is tipped into C, and the dried acid in B is washed into A simultaneously. Denoting the total resulting pressures set up in vessels O and I by h_0 and Δp respectively, the amounts of CO_2 liberated are $h_0 k_0$ and $\Delta p . k_1$. The difference $h_0 k_0 - \Delta p . k_1 = \Delta x'$, is the amount of acid retained by the serum in vessel I under the conditions of this particular experiment.

This amount $\Delta x'$ is equal to Δx only if the quantity of acid in bulb B is exactly equal to x_G, *i.e.* if

$$\Delta p = \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(9).}$$

In choosing the amounts of acid some preliminary experiments with varying amounts of acid in B may be necessary. The amount of acid in D is of course in these estimations kept constant and equal to x_{CO_2} in the main experiment.

If the amount of acid taken is not exactly the same as that in the main experiment, but the conditions approximate fairly closely to those prescribed by equation (9), the observed value of $\Delta x'$ may be corrected for the conditions of the main experiment as follows:

$$\Delta x = \frac{\Delta x'}{\Delta p} . \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(10).}$$

The use of the above principle of calculation of aerobic glycolysis in serum is made possible by the direct determination of x_{CO_2} by the new method, and has the following advantages.

(1) It is not necessary to calculate the retention for CO_2 from an observation of the retention for lactic acid, or *vice versa*.

(2) When both glycolysis and respiration occur together, as for example in tumour tissue, the effect of the lactic acid retention is to cause a change in the retention for CO_2 and *vice versa*, during the experiment. This mutual influence of the two retentions is allowed for in the above direct method of determination.

Accuracy. The probable error to be expected in the value of R.Q. may be conveniently considered on the basis of equations (4 *a*) and (8). Under these simplified conditions (weight of tissue, and constants for carbon dioxide equal in the different vessels) the relationship between the pressures directly observed and the R.Q. is as follows:

$$\text{R.Q.} = \frac{(H_1 - H_0)}{(H_1 - H_0) + h_{B_0} - h_{B_1} - h} . \frac{k_{CO_2}}{k_{O_2}} \qquad \text{.....(11).}$$

In this equation the largest term, $H_1 - H_0$, is present in both numerator and denominator. This is very favourable to the accuracy of the method since any slight error in $H_1 - H_0$, due either to the error in readings themselves

$\pm$ 0·5 mm.), or to slight inequalities in the initial amount of CO_2 in the experimental and control vessels has a negligible effect on the value of R.Q.

The limiting factor in the accuracy is therefore the measurement of h_{B_0} and $(h_{B_1} + h)$. The difference of these two terms which occurs in the denominator of equation (11), is always smaller than $(H_1 - H_0)$, and the accuracy of measuring this quantity is controlled by

(1) accuracy of reading ($= \pm$ 0·5 mm.);

(2) accuracy in pipetting equal amounts of $NaHCO_3$ in the control and experimental vessels;

(3) agreement in bicarbonate content of the two pieces of tissue in the two vessels.

On the basis of the above error in scale-readings, and the experimentally observed accuracy in determining (2) and (3), and assuming a respiration of the order mentioned in this paper, the probable accuracy may be calculated to be 2 to 3 %, or 0·02 in the determination of R.Q. This order of agreement between duplicate determinations of R.Q. on the same tissue has been obtained in a number of experiments, which will form the subject of Part IV of this series.

We wish to express our indebtedness to Prof. O. Warburg, with whom we had the privilege of discussing the problem in the early stages of the development of the method.

We also wish to make acknowledgment to the Halley Stewart Trust Fund for a Scholarship held by one of us (F. Š.).

REFERENCES.

Dickens and Šimer (1930). *Biochem. J.* **24**, 905.
Krebs (1930). *Biochem. Z.* **220**, 250.
Warburg (1925). *Biochem. Z.* **164**, 481.
—— (1930). Metabolism of tumours. (Constable, London.)
—— and Kubowitz (1929). *Biochem. Z.* **214**, 7.

Protocol.

Rat liver in inactivated horse-serum containing 0·2 % glucose. Rat fasted 24 hours. Duration of experiment, 3 hours. Gas-mixture, oxygen with 4·50 % CO_2. Bicarbonate content of serum =461 mm.³ per cc. Hence p_H=7·41. Temperature 38°.

Vessel No.	7	9	10	11	12
Total volume (cc.)	19·73	21·38	21·00	20·16	—
Solutions:					
A (Fig. 1)		1·5 cc. serum			—
B ,,		0·1 cc. HCl			—
C ,,		2·0 cc. $KMnO_4$			—
D ,,		0·2 cc. NaI			—
Weight of tissue:					
Wet (mg.)	80	90	90	90	—
Dry (mg.)	15	18·7	21·2	21·3	—

Gas passed through vessels in bath for 10 mins. Taps then closed and first reading taken after further 5 mins. (t_0).

Readings:

Pressure changes corrected for thermobarometer (mm. Clerici solution of sp. gr. 3·975).

	At t_0 tip B into A	—	—	Tip B into A	—
	$h_{B_1}=+113$	—	—	$h_{B_1}=+111$	—
	At t_1	$h=-30·5$	$h=-34$	—	—
		Tip B into A	—	—	—
		$h_{B_1}=+103·5$	$h_{B_1}=+105·5$	—	—
		Tip D into C (all vessels)			
	$H_0=235$	$H_1=280$	$H_1=286·5$	$H_0=234·5$	$H_{gas}=111$
Calculation:					
Vessel constants					
k_{CO_2}	6·17	6·72	6·60	6·31	—
k_{O_2}	—	5·92	5·84	—	—
k_{O_2}/k_{CO_2}	—	0·889	0·884	—	—
$h_B k_{CO_2}=B$ (mm.³)	697	696	696	700	—
$B_0-B_1=x_b$ (mm.³)	—	3	3	—	—
$(H-H_{gas})\,k_{CO_2}=C$ (mm.³)	764	1136	1157	779	—
$C_1-C_0=x_{CO_2}$ (mm.³)	—	364	385	—	—
$hk_{O_2}-k_{O_2}/k_{CO_2}(x_{CO_2}+x_b)$ $=x_{O_2}$ (mm.³)	—	−508	−540	—	—
Respiratory quotient:	—	0·72	0·71	—	—
Q_{O_2} (mm.³ oxygen respired per mg. tissue per hr.)	—	−9·1	−8·5	—	—

CVIII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

IV. THE RESPIRATORY QUOTIENT IN BICARBONATE-MEDIA.

By FRANK DICKENS and FRANTIŠEK ŠIMER.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

(*Received June 30th, 1931.*)

The experiments described in Part II of this series [Dickens and Šimer, 1930] demonstrated the existence of a definite relationship between the aerobic and anaerobic metabolism of carbohydrate by normal tissues. As has often been pointed out, the figures for total oxygen consumed in respiration and those for anaerobic glycolysis appear to bear no mutual relationship whatsoever [*e.g.* Meyerhof, 1930, p. 178]. When, however, the previous observations were completed by the addition of measurements of the R.Q. and, instead of considering the relationship of the total respiration to the glycolysis, we compared the glycolysis with that fraction of the respiration corresponding to the oxidation of carbohydrate, it was then found that these two sets of values ran parallel in all the types of normal tissue studied. In tumours, on the other hand, there is a definite damage to the oxidation of carbohydrate, so that the amount of carbohydrate oxidised to CO_2 and water is smaller than would correspond with the high value of anaerobic glycolysis in such tissues.

In the above experiments, the tissue was suspended for the purpose of the measurements in a Ringer solution buffered with phosphate. It was considered desirable to ascertain if this relationship is generally applicable, and particularly whether it holds in the case when bicarbonate-media such as serum or bicarbonate-Ringer, are used. For this purpose it was necessary to evolve a method suitable for the measurement of R.Q. under these conditions. The present paper concerns the application of this method to the above problem and the comparison of the R.Q. and respiration of tissues in bicarbonate- and phosphate-media.

It may be stated at once that the conclusions of Part II hold good under the new conditions and are therefore independent of the particular medium used for the experiments. The detailed discussion of the results is left until after the description of the experiments.

Experimental.

The method used for the determination of R.Q., respiration and aerobic glycolysis has been fully described in Part III [Dickens and Šimer, 1931]. The tissue, cut into thin slices (except when, as with the retina or embryo, the intact material is suitable for use without cutting), is suspended in bicarbonate-Ringer solution [Warburg, 1930] or horse-serum, the latter being previously inactivated by heating to 56° for 1 hour. In all experiments glucose (0·2 %) was added to the medium before use. The serum used contained a normal amount of lactic acid (25–30 mg./100 cc.).

It is necessary to repeat here that, for the sake of accuracy in the R.Q., the total oxygen consumption must not fall much below 300 mm.^{3} and, on the other hand, the establishment of diffusion equilibrium is endangered, particularly in experiments in serum, by a more rapid gas exchange than about 100 mm.^{3} per hour. Hence, in general, the experiments lasted for about 3–5 hours; the figures given for respiration and glycolysis are, therefore, the mean values over the whole of this period.

Accuracy of the method.

Table I contains the results of a number of duplicate experiments by the new method, made simultaneously on different slices of the same piece of tissue or, in the case of embryo, by the use of two embryos of the same age. The Table shows that the accuracy of the method is sufficient and is in agreement with that to be expected from the considerations given in the previous paper.

Table I.

Tissue	Serum or Ringer solution	R.Q.
Liver	S	0·72, 0·71
	S	0·73, 0·71
Kidney	R	0·81, 0·82
Testis	R	0·88, 0·87
	R	0·93, 0·92
	S	0·93, 0·92
Brain cortex	R	0·99, 0·99
Chicken embryo	R	0·99, 0·98
Jensen sarcoma	R	0·75, 0·73
	S	0·79, 0·79
Slow-growing sarcoma	S	0·77, 0·77

Results.

The results of all measurements are collected in Table II. The tissues selected are representatives of groups with differing degrees of anaerobic glycolysis. A typical protocol of an experiment, showing the details of an actual measurement and also the method of calculation, was given at the end of Part III.

R.Q. in bicarbonate and in phosphate. In a number of experiments simultaneous duplicate determinations of R.Q. were made on slices from the same

Table II. *Metabolism in bicarbonate and phosphate.*

Specimen no.	Tissue	Bicarbonate-medium: Ringer or serum	$NaHCO_3$ m-mols	CO_2 in gas mixture vols. %	R.Q.	Q_{O_2} (respiration)	$Q^{O_2}_{CO_2}$ (glycolysis)	Phosphate-medium: R.Q. (Same specimen of tissue as in bicarbonate)	Phosphate-medium: Q_{O_2} (Same specimen of tissue as in bicarbonate)
	Liver								
6	Fasted	R	14	1·3	0·55	13·4	2·9	—	—
7	Fed	R	14	1·3	0·86	9·7	2·1	—	—
9	Fasted 12 hrs.	R	14	1·3	0·77	10·7	1·4	0·71 0·68	7·6 6·4
22	Fasted	S	20	4·5	0·72 0·71	9·1 8·5	0·9 0·9	0·65 0·63	9·9 10·2
23	Fasted	S	20	4·5	0·73 0·71	12·7 11·2	1·3 1·6	0·58 0·58	8·6 9·5
35	Fed	S	20	4·5	0·76 0·73	10·0 9·3	2·3 2·6	—	—
36	Fasted	R	20	4·5	0·66 0·64 0·66	13·7 13·9 13·5	4·2 4·3 4·1	0·68 0·68	11·4 11·2
	Kidney								
1	—	R	14	3·2	0·82	18·9	0	—	—
2	—	R	14	1·9	0·78	19·1	0	—	—
18	—	R	25	4·5	0·81 0·82	22·1 20·7	0 0	0·85 0·82	20·7 18·0
19	—	S	20	4·5	0·89	22·8	−6·2	—	—
28	—	S	20	4·5	0·86	22·8	−3·2	0·86 0·87	23·0 26·0
	Testis								
5	—	R	14	—	0·87	10·2	2·8	—	—
12	—	R	25	—	0·88 0·87	8·5 7·3	4·3 4·6	0·88 0·87	7·7 8·6
20	—	R	25	—	0·93 0·92	10·0 10·5	4·9 5·5	0·89 0·91	9·3 9·1
21	—	S	20	—	0·93 0·92	8·4 9·1	2·8 2·6	0·96 0·93	8·5 7·9
	Brain cortex								
3	—	R	14	1·3	1·00	10·6	1·6	—	—
4	—	R	14	1·3	0·99	12·8	1·2	—	—
11	—	R	25	5·0	1·00	16·2	0·3	—	—
15	Young rat	R	25	4·5	0·99 0·99	14·0 14·8	2·5 2·1	—	—
16	Old rat	R	25	4·5	0·96 0·94	15·2 14·4	3·2 2·7	—	—
17	Young rat	S	20	4·5	1·04	13·9	(3·9)	—	—
29	Senile rat	R	25	—	0·95 0·95	10·0 10·0	2·7 3·2	—	—
	Chorion								
10	From embryo of dry wt. 145 mg.	R	25	4·8	0·98	25·4	7·4	—	—
25	From embryo of dry wt. 161 mg.	S	20	4·5	1·02	26·9	−0·9	—	—
	Retina								
31	—	R	20	4·5	1·00	19·5	26·9	—	—
32	—	R	20	4·5	1·03	18·8	19·7	—	—
	Embryo (at 40°. Chicken 4th day)								
33	(Dry wt. 4·10 mg.)	R	20	4·5	0·98	11·3	1·3	—	—
34	(Dry wt. 3·08 mg.)	R	20	4·5	0·99	11·6	3·2	—	—
	Jensen sarcoma								
13	—	R	25	4·5	0·75 0·73	9·5 9·0	8·9 8·9	0·80 0·78	8·5 8·0
26	—	R	25	4·5	0·84 0·77	10·1 10·0	10·4 16·0	—	—
27	—	S	20	4·5	0·79 0·79	8·0 8·8	9·6 11·6	0·79 0·79	8·7 11·1
	Slow-growing sarcoma								
30	—	S	20	4·5	0·77 0·77	5·6 4·3	9·8 7·0	0·88 0·90	5·5 6·3

piece of tissue suspended (*a*) in bicarbonate-Ringer or serum, and (*b*) in phosphate. The compositions of the bicarbonate-Ringer and phosphate-Ringer solutions used were identical in respect to glucose (0·2 %), calcium and potassium, and the concentration of bicarbonate was also equal to that of secondary phosphate. Such control experiments with phosphate-Ringer were not made with those tissues previously shown to have a R.Q. of unity in this medium (brain, embryo, retina, chorion).

Reference to these individual experiments, which are included in Table II, shows that with the exception of liver tissue (see below) there is no systematic difference in the values of R.Q. observed in the two types of media, nor any significant difference in R.Q. due to the use of either serum or Ringer solution, or to the different CO_2 tensions or bicarbonate concentrations shown in the Table. These facts are also fully supported by a comparison of the mean values of R.Q. These figures are given in Table III, in which the mean values obtained in the numerous experiments in Part II are compared with the mean of the figures in bicarbonate-media obtained in the present paper.

Table III.

Tissue	Bicarbonate-medium. Mean R.Q.	Phosphate-medium R.Q. Mean of all previous experiments
Liver	0·71	0·79
Kidney	0·83	0·85
Testis	0·90	0·94
Brain cortex	0·98	0·99
Embryo (chicken)	0·99	1·00
Chorion (rat)	1·00	1·02
Retina	1·01	1·00
Jensen sarcoma	0·78	0·83
Slow-growing sarcoma	0·77	0·94

From Table III it is clear that if the tissues are arranged according to the mean value of R.Q., not only the order of the various tissues in the series is the same, but in addition to this, in the case of each individual tissue, the values of R.Q. in phosphate and bicarbonate respectively differ only slightly. In all normal tissues the difference does not exceed the variation between the measurements on different specimens of the same tissue measured in the same medium. However, it may be significant that in all cases the mean R.Q. in bicarbonate is very slightly below that in phosphate.

In the two types of tumour examined this difference, though in the same direction, is more pronounced. The number of specimens examined as yet is insufficient for any general conclusion to be drawn from this fact, but it is sufficient to note that in all examples the R.Q. isdefinitely below unity, and is in fact lower in bicarbonate or serum than in phosphate.

R.Q. of liver tissue. In a number of experiments in phosphate-media values of R.Q. below the fat-level have been obtained with livers of fasted rats (Table IV). The time since last giving food was in most cases 24 hours. Water was present in the cages throughout. These low quotients are not observed

Table IV. *R.Q. of liver of fasted rats measured in phosphate-Ringer solution, 0·2 % glucose, 38°.*

R.Q.	Q_{O_2}
0·61	8·9
0·57	7·1
0·59	7·8
0·62	8·4
0·62	8·7
0·65	9·9
0·63	10·2
0·58	8·6
0·58	9·5

in the case of animals which have been fed during a period of about 12 hours preceding the experiment. Consequently, they were not encountered in the series of rats used for the experiments in Part II, since in these experiments all the animals were fed either on the day of the experiment or on the evening of the previous day.

When similar experiments with fasted and with fed animals were made in bicarbonate-Ringer solution and in serum, it was found that the measurements in Ringer solution resembled those in the phosphate-medium, in that values of R.Q. below the physiological level were obtained with fasting animals. In serum, on the other hand, the value of R.Q. was within the normal limits and corresponded with the value found for the oxidation of fats.

R.Q. of brain tissue. In some of the experiments with cerebral cortex of rats, quotients a little below unity were obtained (Table II). It was noted that in all these experiments old rats had been used; in one example (specimen 29), R.Q. = 0·95, a very old animal aged about 2 years was used. The results suggest a tendency for the R.Q. to fall a little below the carbohydrate level with advancing age of the rat in the case of brain tissue.

Measurements of respiration (Q_{O_2}) *by the new method.* Reference to the figures in Table V shows that the extent of respiration as measured by the direct method used in this paper is in agreement with that found by Warburg [1930] by the use of his bicarbonate method. In view of the fact that our

Table V.

Tissue	Q_{O_2} This paper (mean value)	Q_{O_2} Warburg [1927]
Kidney	21·1	21
Liver	11·3	12
Testis	8·4	12
Brain	13·2	11
Chorion	26·2[1]	14·4[2]
Retina	19·2	17[3], 31[4]
Embryo	11·5[5]	10[5]
Jensen sarcoma	9·2	9

[1] Embryo dry weight 150 mg.
[2] Embryo dry weight 15·4 mg. [Negelein, 1925].
[3] Warburg, Negelein and Posener [1924].
[4] Warburg, Posener and Negelein [1924].
[5] Chicken embryo, weights 3·5 and 1·7 mg. respectively (dried at 110°).

measurements extend over a period of 3–5 hours, and are therefore not maximal but mean values over this period, this agreement is quite satisfactory, and shows that in most cases the respiration remained nearly constant over this long period. In one instance, that of chorion, the respiration observed on two separate specimens was found to be considerably higher than that given by Negelein [1925]. The latter, however, refers to the membrane obtained from smaller embryos than those which provided the material for our experiments.

Measurements of glycolysis ($Q^{O_2}_{CO_2}$). The new method has the advantage over the phosphate method that it permits the simultaneous measurement of aerobic glycolysis, as well as respiration and R.Q., on the same piece of tissue. The method is the only one suitable for the detection of slight aerobic formation of acid, either in Ringer solution or serum. From Table II it will be seen that, under the conditions of these experiments, nearly all normal tissues produce small amounts of acid, and this is true whether the medium for the experiment be serum or bicarbonate-Ringer. It will be noticed, for instance, that in the experiment with testis the acid production diminishes but does not disappear completely even in serum.

The only exceptions to the above statement in the series of tissues studied, were kidney and chorion. With kidney in Ringer solution the bicarbonate content in all four experiments was, within the limits of experimental error, quite unchanged during the whole period of the experiment. In serum, on the other hand, kidney caused a definite increase in the content of bound carbon dioxide over and above that corresponding to the retention of respiratory carbon dioxide by the serum (specimens 19 and 28, Table II). In the case of chorion, the appreciable aerobic glycolysis found in Ringer solution not only disappears in serum [Negelein, 1925] but there is even a slight consumption of fixed acid in the latter medium (specimen 25).

It should be remarked that knowledge of the nature of the traces of acid produced aerobically is at present inadequate. Owing to the small amount of acid formed in such cases it is very difficult to control the manometric observations on this point by reliable chemical methods of analysis, and it cannot yet be definitely assumed that all the acid formed is lactic acid in the case of the feebly glycolysing normal tissues.

A further complication in interpreting these data for the aerobic glycolysis of normal tissues, is the possibility of a portion of the acid formed being neutralised by a simultaneous ammonia-liberation by the tissue. For instance, in the case of kidney even in the presence of glucose an amount of ammonia (0·9 mm.^{3} per mg. tissue per hour [Warburg, 1930]) comparable with the figures for aerobic glycolysis found in the other examples of normal tissues in Table II (*e.g.* in liver tissue), appears in the solution in which the kidney tissue is suspended.

Turning to the highly glycolysing tissues such as the retina and tumours, it will be seen from Table II that the figures for aerobic glycolysis are lower

than those given by Warburg's bicarbonate method. This is due to the extended period of the experiments and the consequent appreciable lowering of the bicarbonate content of the Ringer solution or serum.

It is not possible in such cases to combine maximum accuracy in determining R.Q. with determination of glycolysis under optimal conditions. In highly glycolysing tissues separate experiments of short duration, such as those of Warburg [1930], must be used for obtaining a standard value of $Q_{CO_2}^{O_2}$.

Discussion.

Effect of medium on respiration and R.Q.

Phosphate- and bicarbonate-Ringer solutions. As was described in Part II, the extent of the respiration of surviving tissues does not suffer when the bicarbonate buffer in the medium is replaced by phosphate. This fact has also been well recognised in work on isolated muscle [Meyerhof, 1930, p. 53], and it is only in the case of other tissues that the suitability of phosphate appears to be questioned. The experiments described in the present paper show that the R.Q. is also very nearly the same in bicarbonate-media as in the simple phosphate-buffered Ringer solution previously used. Hence the respiration is both quantitatively and also, at least as far as concerns the type of metabolites oxidised, qualitatively independent of the nature of the buffer solution in which the tissue is suspended. As far as the available evidence shows, the state of the tissue in these different media seems to differ only in respect of its behaviour under certain quite special conditions. A much discussed example of this is provided by the action of cyanide in the two media [Dixon and Elliot, 1929; Alt, 1930; Warburg, 1931].

Serum and Ringer solution. The evidence previously advanced in favour of the use of serum as a medium for measurement of tissue metabolism was the effect on the aerobic glycolysis [Warburg, 1930]. In our experiments there is in most cases a tendency for aerobic glycolysis to be diminished in serum (*e.g.* in the case of testis), a fact which may be interpreted as indicating some damage to the tissue in the simple salt solution. The best example is given by chorion in which the appreciable aerobic glycolysis observed in Ringer-bicarbonate disappears when the tissue is suspended in serum (specimen 25). A new instance observed in the present paper is the effect of serum on the abnormally low R.Q. observed with the livers of starved animals in simple salt solutions. Here the damage to respiration in Ringer solution is shown by a value of R.Q. which may be compared with that of the atypical type of oxidation found in mechanically damaged tissue and in tissue extracts. That the effect of serum in this case is not to be explained by consumption of lactic acid contained in normal physiological concentration in this medium is shown by the fact that in all three experiments in serum (specimens 22, 23, 35), there was no disappearance of fixed acid from the medium (see, for instance, protocol at end of Part III) either with fasted or with fed animals. The con-

centration of lactate in the serum, however, was sufficient to cause a marked consumption of this substance by kidney tissue in serum (specimens 19 and 28).

It is proposed to investigate more fully this question of the consumption of lactic and pyruvic acids by tissues, for which purpose the method used in this paper is particularly suitable. One of the problems awaiting solution is the discrepancy between the above findings and the utilisation of lactic acid by liver *in vivo*. *In vitro*, in the presence of a large concentration of lactate, there is a definite increase in the respiration with accompanying consumption of lactate [Meyerhof and Lohmann, 1926] and increase of the value of R.Q. [Dickens and Šimer, 1930] of liver tissue above the level in glucose. Meyerhof and Lohmann [1926] also observe an action of serum in increasing the respiration of liver tissue which they attribute to the same cause, namely the presence of lactate in the serum. Our experiments on this point do not support this view, since no disappearance of lactic acid was observed, at least from serum of normal lactic acid content and containing an adequate supply of glucose. The same authors give some low figures for R.Q. of the liver of a starved rat in serum. The serum used by them however was acidified and the CO_2 removed by evacuation.

Relationship between respiration and glycolysis. The values in Table III and the agreement of individual comparisons in phosphate and bicarbonate (Table II) are sufficient to establish the correctness of the values of R.Q. previously measured. All quotients not only in normal tissues, but also in tumours, lie between the limits corresponding to the complete oxidation of normal foodstuffs.

The regular relationship previously found between the oxidative and anaerobic metabolism of carbohydrate in normal tissues is confirmed by measurements made under physiological tension of CO_2 in serum or bicarbonate-Ringer solution. Under the same conditions the R.Q. of tumour tissue is definitely below the carbohydrate level, and indicates a damaged oxidation of carbohydrate by such tissue.

The relationship found for other normal tissues can also be extended to the case of resting muscle. The value of R.Q. is below the carbohydrate level in this tissue (*e.g.* for diaphragm of the mouse R.Q. = 0·84, mean of six experiments in phosphate Ringer with glucose), a fact which is in accordance with the value of anaerobic glycolysis ($Q^{N_2}_{CO_2} = 3$–4). Unfortunately, similar measurements on working mammalian muscle are not available. A more general illustration of this relationship is provided by the developing embryo. As was shown by Negelein [1925] the glycolytic power of embryonic tissues falls regularly during development. Our measurement of R.Q. of young (4–5 day) chicken embryos shows that the oxidative metabolism of such embryos is a purely carbohydrate one. Needham [1931] in his exhaustive discussion of the probable nature of foodstuffs utilised during embryonic development, regards the development of the chick embryo as being divided into three periods of which the first, corresponding to the first five or six days

of incubation, is a period of carbohydrate oxidation; the second a period of protein utilisation and in the last stage the energy is derived mainly from the oxidation of fat. Corresponding with the disappearance of free glucose in the first eight days, an accumulation of lactic acid was found by Tomita [quoted by Needham, 1931]; this subsequently falls to a low level. All these facts are in complete agreement with our general conclusion that the replacement of an oxidative metabolism of carbohydrate by one of fat is associated with a corresponding lowering of the glycolytic power; the oxidative and glycolytic cleavage of carbohydrate proceeding hand in hand in this as in other cases.

SUMMARY.

1. The method for the measurement of R.Q., respiration and aerobic glycolysis previously described (Part III) has been applied to a series of normal and tumour tissues.

2. The accuracy of the method is shown by the good agreement between duplicate determinations.

3. No significant difference has been observed in the values of R.Q. and respiration measured in salt solutions buffered (*a*) by bicarbonate under physiological tension of CO_2, or (*b*) by phosphate in a CO_2-free atmosphere.

4. The respiratory quotients of the tissues of fed animals are in all cases within the limits corresponding to the oxidation of normal foodstuffs.

5. In fasting animals, the R.Q. of liver tissue in simple salt solutions containing glucose falls below the physiological level. It is restored to the fat level when the measurement is made in serum.

6. The conclusions derived from the experiments in phosphate-media previously reported (Part II) are established for bicarbonate-media also, and some new examples of the relationship between oxidation of carbohydrate and its glycolytic cleavage are brought forward.

We wish to thank Prof. S. Russ and Prof. J. McIntosh for tumour material used in these experiments.

One of us (F. D.) is working as a member of the scientific staff of the Medical Research Council and the other (F. Š.) is in receipt of a scholarship from the Halley Stewart Trust Fund.

REFERENCES.

Alt (1930). *Biochem. Z.* **221**, 498.
Dickens and Šimer (1930). *Biochem. J.* **24**, 1301.
—— —— (1931). *Biochem. J.* **25**, 973.
Dixon and Elliot (1929). *Biochem. J.* **23**, 812.
Meyerhof (1930). Die chemischen Vorgänge im Muskel. (Springer, Berlin.)
—— and Lohmann (1926). *Biochem. Z.* **171**, 381.
Needham (1931). Chemical embryology (in press). (Univ. Press, Cambridge.)
Negelein (1925). *Biochem. Z.* **165**, 122.
Warburg (1927). *Biochem. Z.* **184**, 484.
—— (1930). Metabolism of tumours. (Constable, London.)
—— (1931). *Biochem. Z.* **231**, 493.
—— Negelein and Posener (1924). *Klin. Woch.* **3**, 1062.
—— Posener and Negelein (1924). *Biochem. Z.* **152**, 309.

CIX. GROWTH AND CHEMICAL COMPOSITION OF OVUM OF FUNCTIONING FOWL'S OVARY (*GALLUS DOMESTICUS*).

By ALEXIS LAWRENCE ROMANOFF.

From the Laboratory of Experimental Embryology, Cornell University Agricultural Experiment Station, Ithaca, New York, U.S.A.

(*Received April 24th, 1931.*)

The rate of growth of the ovum of *Gallus domesticus* was divided by the earliest workers into several more or less distinct periods. His [1868] distinguished by microscopic appearance four periods, while D'Hollander [1904] distinguished, on the basis of nuclear phenomena, three periods. Later Riddle [1916] suggested that there might be only two phases of the growth of the ovum: (1) from primary oöcytes up to 3 mm. in diameter, and (2) from 3 mm. to 6 mm. in diameter. At the end of the second period the rate of growth of the ovum is marked by a rapid deposition of yolk. Within 5 to 8 days the ovum reaches full maturity and is ready for exclusion. The latter period of growth or maturation of the ovum is caused by rapid assimilation of food materials, which constitute the chemical elements of the yolk proper. These chemical elements have probably never been determined quantitatively in successive stages of maturation as well as during the early growth period of the ovum. To throw some light on this problem a series of analyses has been performed.

The ova used were only from White Leghorn hens of known history. The experiments were conducted in the spring months (May and June) when the ovaries of possibly all egg-laying animals are under natural stimulus for egg production [Hoffmann, 1892]. A hen was killed at the time when an egg could be detected in the lower portion (uterus) of the oviduct, to provide uniform conditions in respect to the size of all ova in the examined ovaries. The length of oviducts was found to be on an average 59 cm., which corresponds with the results of Buckner and Martin's [1929] study of laying hens.

The data on the growth of the fowl's ovum are shown in Table I. As already suggested, these indicate growth periods, shown by the weight and shape of the ovum. Riddle [1916] and Stieve [1918] demonstrated that the ovum is almost a spherical body. However, our data on the shape of ova have been obtained by measuring the height (with spherometer) and width (with micrometer) of an ovum laid on a plane surface. The ratio of height to width might therefore be different from that described by the above workers. It is an index of elasticity of the vitelline membrane of the ovum.

Table I. *Growth of fowl's ovum.*

Phases of growth	Series of ova No.	Size of ovum: Average weight g.	Size of ovum: Dry matter content %	Shape of ovum: Height mm.	Shape of ovum: Width mm.	Shape of ovum: Ratio H/W Index
After laying	—	17·650	52·36	14·5	39·0	0·37
Before laying	—	17·076	52·76	16·0	35·5	0·45
Maturation phase	1	15·029	54·54	23·5	32·5	0·72
	2	10·782	53·20	20·5	29·5	0·69
	3	5·572	51·05	17·0	23·5	0·72
	4	2·287	48·15	12·0	17·5	0 69
	5	0·608	39·30	7·5	10·5	0·71
	6	0·250	18·57	6·0	8·5	0·71
	7	0·199	17·60	5·5	7·5	0·73
Second phase of growth	8	0·169	15·18	5·25	7·0	0·75
	9	0·157	14·45	5·0	6·5	0·77
	10	0·144	14·25	4·25	5·5	0·77
	11	0·128	14·12	3·5	4·5	0·78
	12	0·122	13·47	3·25	4·0	0·81
	13	0·115	11·90	3·0	3·75	0·80
	14	0·095	14·43	3·0	3·5	0·86
	15	0·089	13·58	3·0	3·5	0·86
	16	0·083	11·32	3·0	3·5	0·86
	17	0·071	15·31	3·0	3·5	0·86
	18	0·065	13·31	3·0	3·5	0·86
	19	0·063	12·80	3·0	3·5	0·86
	20	0·058	—	3·0	3·5	0·86
	21	0·045	—	3·0	3·5	0·86
	22	0·042	—	2·75	3·25	0·85
First phase of growth	23	0·035	—	2·75	3·0	0·92
	24	0·016	—	2·75	3·0	0·92
	25	0·013	—	2·5	2·75	0·91
	26	0·012	—	2·5	2·75	0·91
	27	0·012	—	2·5	2·75	0·91
	28	0·012	—	2·5	2·75	0·91

This index is noticeably high in small ova, and rapidly declines after ovulation, because the yolk, by absorption of moisture from the surrounding albumin, enlarges in size, becomes watery and thus weakens the elasticity of the vitelline membrane. The percentage of dry matter rapidly increases during maturation, reaching its highest point in a large ovum. Soon after ovulation this value drops to that which is usually found in fresh eggs.

The density of the contents of large ova corresponds with the percentage

Table II. *Chemical composition of fowl's ovum.*

Series of ova	Density $\frac{W_1}{W} D$	Calcium (CaO): Content g.	Calcium (CaO): % of wet weight	Fat (ether extract): Content g.	Fat (ether extract): % of wet weight	Fat (ether extract): Iodine value (Wijs)	Fat (ether extract): Refractive index	p_H
After laying	1·03181	0·412	2·33	5·363	30·38	72·58	1·4685	5·985
Before laying	1·03187	0·410	2·40	5·148	30·15	(71·95)	1·4679	5·968
In ovary:								
1st ovum	1·03448	0·390	2·59	4·294	28·57	73·35	1·4685	5·900
2nd ,,	1·03372	0·208	1·93	3·161	29·32	68·33	1·4675	5·917
3rd ,,	1·03128	0·134	2·40	1·710	30·69	60·95	1·4679	6·027
4th ,,	—	0·091	3·98	0·652	28·51	—	1·4672	6·137
5th ,,	—	0·029	4·77	—	—	—	—	6·289

of dry matter (Table II). The content of calcium, as calcium oxide, gradually increases up to the time of ovulation with a slight change in the percentage composition. Fat (ether extract) content increases but without change in the percentage composition. There is an indication of a slight change in the iodine value, and no change whatsoever in the refractive index. Hydrogen ion concentration shows little if any change.

Conclusions.

The growth phases and particularly the maturation phase of the fowl's ovum are marked by a very rapid increase in the percentage of dry matter, reaching the highest point in a mature ovum.

Calcium and fat contents of the ovum increase proportionately with the advance of maturation.

Density and p_H of ovum, iodine value and refractive index of the extracted fat show only slight changes during the maturation phase.

REFERENCES.

Buckner and Martin (1929). *Amer. J. Physiol.* **89**, 164.
His (1868). Untersuchungen über die erste Anlage des Wirbelthierleibes. Die erste Entwickelung des Hühnchens im Ei (Leipzig).
Hoffmann (1892). *Arch. Neerl. Sci. Nat.* **17**, 168.
D'Hollander (1904). *Arch. Anat. Micr.* **7**, 117.
Riddle (1916). *Amer. J. Physiol.* **41**, 387.
Stieve (1918). *Arch. Entw.-mech. Organ.* **44**, 530.

CX. A NEW SYNTHESIS OF METHIONINE.

By GEORGE BARGER
and THEODORE EDWIN WEICHSELBAUM.

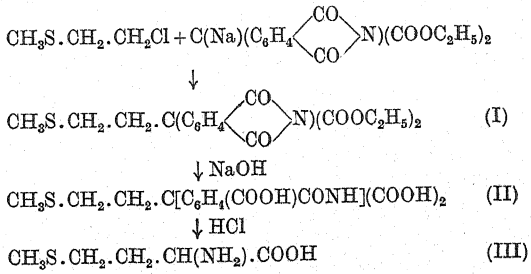
From the Department of Medical Chemistry, University of Edinburgh.

(*Received May 21st, 1931.*)

The Strecker synthesis of methionine by Barger and Coyne [1928] served its purpose in establishing the constitution of the amino-acid, but the yield (6 %, based on the β-methylthiolpropaldehyde employed) did not help to make the substance more easily available than the natural product, which is very troublesome to isolate. A second synthesis by Windus and Marvel [1930] (bromination of β-methylthiolethylmalonic acid) did not result in a greatly improved yield (7 % based on the α-methylthiol-β-chloroethane employed), probably owing to the oxidation of the methylthiol group.

In order to render the amino-acid more readily available for metabolic studies and for investigation of its optical properties, we thought it desirable to explore other possible syntheses with a view to obtaining increased yields. The synthesis described in this paper avoids the main difficulties of the preceding ones, namely the use of a rather inaccessible intermediate, acrolein, and the oxidation of the methylthiol group by bromination. By its use we have been able to accumulate a considerable supply of methionine and at present we are engaged in its resolution. It will be recalled that the method for the isolation of the naturally occurring substance involves the use of alkali under conditions which probably lead to at least partial racemisation.

Following the lines which led Sørensen [1903–6] to the synthesis of α-amino-adipic acid and other amino-acids, ethyl sodiophthalimidomalonate was made to react with α-methylthiol-β-chloroethane. On hydrolysis of the resulting compound (I), first with alkali to the phthalamidomalonic acid (II) and then with acid, methionine (III) was obtained in a yield of 58 % (based on the α-methylthiol-β-chloroethane employed). The reactions are represented by the following equations:



Experimental.

α-Methylthiol-β-chloroethane. 440 g. of methyl *iso*thiocarbamide sulphate, prepared according to the method of Arndt [1921] in a yield of 95 %, were hydrolysed by 670 cc. of 5*N* sodium hydroxide and the methylmercaptan obtained was directly absorbed in a solution of 5·5 g. of sodium in 1185 cc. of commercial alcohol. This solution was heated to boiling and 207 g. of ethylene chlorohydrin were added in such a manner that the solution continued to reflux gently. After the addition of the ethylene chlorohydrin the solution was boiled for 10 hours. It was found inadvisable to distil the alcohol from the solution since the large amount of sodium chloride present caused considerable danger of breakage from bumping. The solution was centrifuged and the salt shaken up with successive quantities of absolute alcohol until it retained no appreciable odour of the thiol compound. The alcoholic solution of the thiol compound was then distilled *in vacuo*, the yield of α-methylthiol-β-hydroxyethane being 126 g. of a product boiling at 59°–70°/16 mm. (51 % of the theory based on the ethylene chlorohydrin employed).

The α-methylthiol-β-hydroxyethane was chlorinated by the method of Bennett [1929]. 318 g. of thionyl chloride were dissolved in 700 g. of dry chloroform and the solution was added drop by drop to the dimethylaniline solution of the thiol compound. The solution was kept at room temperature for 2 hours and then heated for 20 minutes on the steam-bath. It was then washed three times with concentrated hydrochloric acid and twice with water, dried over calcium chloride, filtered, and freed from chloroform by distillation. The residue of crude chlorosulphide was distilled *in vacuo*. The yield was 115 g. of a product boiling at 50°/25 mm. (70 % of the theory).

Ethyl sodiophthalimidomalonate [Sørensen, 1905]. To a solution of 4·6 g. of clean sodium in 150 cc. of absolute alcohol (freshly distilled over sodium) at about 60°, 63 g. of ethyl phthalimidomalonate [Sørensen, 1903–6; Organic Syntheses, 1927] were added and the mixture was shaken. The sodium compound, which crystallised on cooling in ice-water, was filtered quickly at the pump, washed twice with absolute alcohol and twice with absolute ether and dried in a vacuum desiccator over sulphuric acid. The yield was 67 g., and by neutralisation of the mother-liquors with hydrochloric acid 5·5 g. of the ester were recovered. A sample of the sodium compound was dried to constant weight at 140°/10 mm., and in 6 hours lost 16·8 % of its weight ($1\frac{1}{2}$ molecules of alcohol of crystallisation require 17·43 % loss); the colour of the substance changed on drying from a rather deep orange-yellow to a very light yellow. This colour change can be used as an index of the completion of the drying of large quantities of the compound, which is essential if one wishes to obtain the highest yield possible in the subsequent step, and this was accomplished by heating in a bolt-head flask in an oil-bath at 145–155°/12 mm. with occasional shaking for 8 hours or until the change of colour of the substance to light yellow was complete.

(Found: 7·036 % Na. Calculated for $C_{15}H_{14}O_6NNa$: 7·04 % Na.)

In order to avoid the inconvenience of having to rid the substance of its alcohol of crystallisation, the method of Dunn and Smart [1930] may be used.

Ethyl β-methylthiol-α-phthalimidoethylmalonate (I). 85 g. of the ethyl sodiophthalimidomalonate were placed in a 250 cc. bolt-head flask fitted with a 3-holed rubber stopper for condenser, thermometer and stoppered glass tube for sampling; 43 g. of α-methylthiol-β-chloroethane (60 % excess) were added and the flask was heated in an oil-bath at 150° until the reaction mixture was no longer alkaline to turmeric paper (in this case the time was 1 hour and 45 minutes). The excess of the chloro-compound was distilled off *in vacuo* (after redistillation 12 g. of a pure product were obtained). The residual yellow oil was mixed with warm water and poured into an evaporating dish where it almost immediately crystallised. The crystalline substance was again extracted with water to remove any remaining sodium chloride and recrystallised from the smallest quantity of alcohol possible. It formed white prisms having M.P. 67° (uncorr.). The yield was 79 g. (74 % of the theory, based on the chloro-compound used).

(Found: 56·96 % C, 5·52 % H, 8·53 % S. Calculated for $C_{18}H_{21}O_6NS$: 57·14 % C, 5·58 % H, 8·44 % S.)

β-Methylthiol-α-phthalamidoethylmalonic acid (II). 25 g. of the ester were dissolved in 30 cc. of alcohol in a 200 cc. bolt-head flask, placed on a boiling water-bath and 70 cc. of 5*N* sodium hydroxide were added. The cloudy liquid was heated until a sample gave a clear solution when diluted with water. The sodium salt of the acid is insoluble in an excess of sodium hydroxide but very soluble in water. For the preparation of methionine it was unnecessary to isolate the free acid, though this was actually done. After the hydrolysis was completed the solution was cooled to 0° in an ice-salt mixture and carefully neutralised with 0·2 *N* hydrochloric acid to Congo red paper. To this solution, still at 0°, 75 cc. of 5*N* hydrochloric acid were added carefully, during which process the acid began to separate in the form of white crystals. In order to complete the salting-out 60 cc. of concentrated hydrochloric acid were added slowly. The white crystalline mass was then filtered at the pump and freed from salt by trituration with ice-cold water. The yield was 21·5 g. and 0·5 g. more was obtained by working up the mother-liquors (97 % of the theory). M.P. 142–143° (uncorr.).

(Found: 49·55 % C, 4·78 % H, 9·56 % S. Calculated for $C_{14}H_{15}O_7NS$: 49·27 % C, 4·40 % H, 9·38 % S.)

Methionine (III). 21·5 g. of the above acid were suspended in 350 cc. of water in a litre flask which was then placed on a boiling water-bath. 40 cc. of concentrated hydrochloric acid were added. Almost immediately carbon dioxide was given off and the substance began to go into solution. The heating was continued for about 1½ hours and 200 cc. more of concentrated hydrochloric acid were added which did not produce a further precipitate. Heating was continued for 45 minutes to complete the reaction. The water and acid were then removed by distillation first at ordinary pressure and then *in vacuo*.

The residue was taken up in warm water, 18 cc. of pyridine were added and three volumes of hot alcohol. On cooling the methionine separated as white crystals. The methionine was then suspended in 200 cc. of boiling absolute ether in order to remove any remaining phthalic acid, filtered at the pump and dried in a vacuum desiccator over sulphuric acid. M.P. 279°-280° (uncorr.). The yield was 8 g. (81 % of the theory based on the α-methylthiolphthalamidoethylmalonic acid employed, or 58 % of the theory based on the α-methylthiol-β-chloroethane).

(Found: 40·47 % C, 7·29 % H, 21·46 % S. Calculated for $C_5H_{11}O_2NS$: 40·23 % C, 7·43 % H, 21·50 % S.)

Methionine methyl ester hydrochloride. A suspension of 2 g. of methionine in 125 cc. absolute methyl alcohol, cooled in an ice-salt mixture, was saturated with hydrogen chloride for 2 hours. The alcohol and hydrochloric acid were then distilled off *in vacuo*. The residue was taken up in acetone from which it crystallised after 1 day in a vacuum desiccator. The ester hydrochloride was filtered off and washed with a small quantity of absolute ether. The yield was 1·9 g. (71 % of the theory) of white needle-like crystals. M.P. 135°.

(Found: 17·74 % Cl. Calculated for $C_6H_{14}O_2NSCl$: 17·59 % Cl.)

We gratefully acknowledge a grant from the Moray Fund of Edinburgh University which partially defrayed the expenses of this investigation.

REFERENCES.

Arndt (1921). *Ber. deutsch. chem. Ges.* **54**, 2236.
Barger and Coyne (1928). *Biochem. J.* **22**, 1417.
Bennett (1929). *J. Chem. Soc.* 2567.
Dunn and Smart (1930). *J. Biol. Chem.* **89**, 46.
Organic Syntheses (1927), **7**, 78 (New York).
Sørensen (1903–6). *Compt. Rend. Trav. Lab. Carlsberg*, **6**, 1.
—— (1905). *Z. physiol. Chem.* **44**, 448.
Windus and Marvel (1930). *J. Amer. Chem. Soc.* **52**, 2575.

CXI. THE VITAMIN D PROBLEM.
II. OPTICAL ROTATION OF VITAMIN D.

By ENGBERT HARMEN REERINK and AART VAN WIJK.

Natuurkundig Laboratorium der N. V. Philips' Gloeilampenfabrieken Eindhoven-Holland.

(*Received June 2nd, 1931.*)

The most simple reaction, that can take place when ergosterol is irradiated with ultraviolet rays, is, of course, Ergosterol → L, *i.e.* the ergosterol is transformed into one single substance, L.

In a previous paper [Reerink and van Wijk, 1929] we have been able to show that under certain conditions the reaction proceeds in such a way that the resulting phenomena are in full agreement with this reaction scheme. This was concluded from a study of the changes in the absorption spectrum, the necessary conditions being:

(1) the light used for the irradiation must not contain rays of wave-length less than 284 $m\mu$[1]);

(2) the irradiation has to be stopped before more than about 50 % of the ergosterol has been transformed;

(3) oxygen must be completely absent;

(4) the solution must be vigorously stirred.

This result was of some importance, since the preparations obtained by irradiation of ergosterol under these conditions show intense antirachitic activity, the degree of activity being proportional to the degree of transformation of the ergosterol [Everse and van Niekerk, 1931, 1, 2]. Therefore, if indeed a single substance is formed under these conditions, this substance must be vitamin D. This conclusion being very important, we have tried to prove its correctness in an independent way, *viz.* by the measurement of the changes in the optical rotation on irradiation.

Experimental.

Since the complete absence of oxygen is a very important condition for the reproducibility of experiments on the irradiation of ergosterol, we have taken care to perform all operations *in vacuo*. In order to be able to do so, we have made extensive use of an implement well known in vacuum technique, the working of which will be easily understood from Fig. 1 *a*. In this way it is quite easy to connect an evacuated vessel with another one,

[1] Formerly we put the limit at 275 $m\mu$, but since then we have found it better to cut off at a somewhat greater wave-length [see Reerink and van Wijk, 1931].

which in its turn can be evacuated and sealed off from the pump; by breaking the partition-wall by means of an iron ball, operated magnetically, the communication between the two vessels is brought about. Thus it is possible *e.g.* to distil a liquid from one vessel into another, or to recrystallise a substance from a solvent and filter off the crystals with a sealed-in filtering disc of sintered glass *etc.*, all operations being performed under air-free conditions.

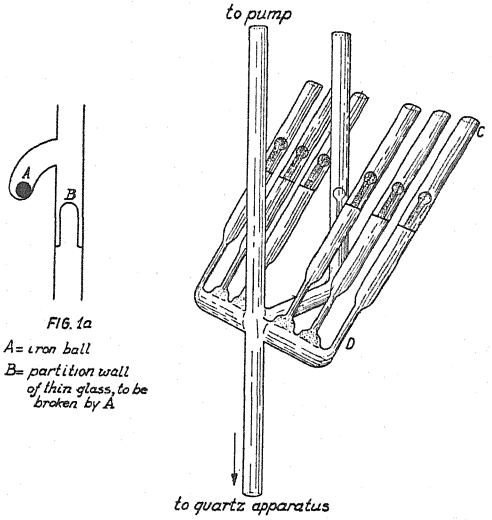


FIG. 1a
A = iron ball
B = partition wall of thin glass, to be broken by A

Fig. 1 *b*.

The solutions were irradiated in an all-quartz apparatus, containing an electromagnetic stirrer and bearing a device for withdrawing samples. The working of the latter will be understood from Fig. 1 *b*: a small part of the solution to be examined can be decanted into one of the sample-tubes, *C*; the tube is then sealed off at the capillary part, *D*, and can be used for further examination. A polarimeter-tube was directly sealed to the apparatus.

The ergosterol had been recrystallised from alcohol-benzene [Bills and Honeywell, 1928] and proved to be quite pure, $[\alpha]_D^{18°}$ being $-$ 134·0° in chloroform. Pure peroxide-free ether was used as a solvent. The whole apparatus was thoroughly evacuated until the pressure of non-condensible gas was less than 0·001 mm., and then sealed off. The solution was irradiated with a quartz mercury arc through a filter of xylene-alcohol, cutting off all radiation below 284 $m\mu$. After a suitable period of irradiation, several readings of the rotation for the yellow sodium line were taken with a Schmidt and Haensch polarimeter; then one of the sample tubes was partially filled with solution and carefully sealed off from the rest of the apparatus. The degree of transformation of the ergosterol was estimated by the digitonin method in the sample withdrawn. With the rest of the solution the irradiation was continued, *etc.*

Experimental results.

(*a*) *Rotation.*

The results of three different series of experiments are shown in Table I, in which the first column gives the time of irradiation in hours, the second the % quantity of unchanged ergosterol as found with the digitonin method, and the third the specific rotation of the irradiated preparation.

Table I.

Time of irradiation in hours	% ergosterol	Rotation measured	Rotation calculated
0	100·0	−105·2°	
1	93·9	−95·8	−94·5°
2	90·7	−87·5	−89·2
4	79·6	−71·3	−70·1
6	70·9	−56·9	−55·1
$8\frac{1}{2}$	60·2	−40·5	−36·8
0	100·0	−105·2	
$\frac{1}{4}$	96·8	−100·4	−99·5
$3\frac{1}{4}$	77·7	−68·7	−66·9
0	100·0	−105·2	
1	94·0	−94·3	−94·8
2	90·1	−85·2	−88·1
4	78·6	−70·0	−68·4
6	72·0	−55·3	−57·0
9	62·2	−38·4	−40·2
13	52·9	−21·5	−24·2

More illuminating is Fig. 2, where the measured values of the rotation from the three series of experiments are plotted against the relative amounts of unchanged ergosterol. This figure indicates clearly, that the points fall on

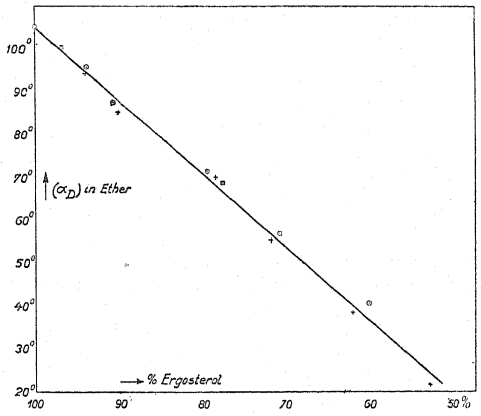


Fig. 2.

a straight line, the deviation being of the order of magnitude of the experimental errors. Now this behaviour is exactly what must be expected if the reaction-scheme of p. 1001 holds true.

If α_E and α_L are the specific rotations of ergosterol and of the reaction product, then, if x % of the ergosterol have been left unchanged, the measured rotation must be

$$\alpha_m = x.\alpha_E + (100-x)\,\alpha_L,$$

or

$$\alpha_m = x.(\alpha_E - \alpha_L) + 100.\alpha_L,$$

giving a linear relation between α_m and x.

Putting the experimental parameters of the line in Fig. 2, equal to the theoretical parameters of the above formula, we obtain for $\alpha_L = +\,66{\cdot}7° \pm 2{\cdot}7°$ (in ether).

By means of this value for the specific rotation of the reaction-product, and of that of ergosterol, the corresponding rotation can be calculated for every degree of transformation (second column in Table I). These calculated rotations are shown in the fourth column of Table I; the deviation of their values from the experimental values prove to be within the limit of experimental error.

It may be concluded from the foregoing, that, just as in the experiments on the absorption spectrum, the experimental facts are in complete harmony with the assumption that the first long-wave reaction-product of ergosterol is a single substance. Still, as was already pointed out in an earlier paper, both series of experiments may also be explained by a more complex hypothesis, *viz.*, that several substances are formed simultaneously in a constant proportion. As up to the present in our experiments we have never found irrefutable facts that could not be explained by the former assumption, which moreover has the advantage of its very simplicity, we are inclined to consider the individuality of the substance L as practically certain.

(*b*) *Rate of the photochemical reaction.*

From the results of these experiments, an interesting calculation can be made concerning the velocity of the reaction: Ergosterol → L, under our experimental conditions.

Since the concentration of the solution is so high that practically all active radiation is absorbed, the rate of disappearance of the ergosterol would be constant, if the intensity of the radiation were kept constant and if the ergosterol were the only absorbent. Now for practical purposes the intensity of the radiation remains constant in one series of experiments, but the ergosterol is not the only absorbent, as the vitamin D, which is formed, also shows an absorption in the same range of wave-lengths. Under these circumstances the incident radiation is divided between the ergosterol and the vitamin, and we may safely assume that the ratio in which the radiation is absorbed by the separate substances is

$$I_{\text{erg}}/I_{\text{vit}} = (\epsilon_{\text{erg}}\, C_{\text{erg}})/(\epsilon_{\text{vit}}\, C_{\text{vit}}),$$

where I_{erg} and I_{vit} are the intensities of the radiation absorbed by ergosterol and the vitamin respectively, ϵ_{erg} and ϵ_{vit} are the absorption indices and

C_{erg} and C_{vit} the concentrations. Strictly, this equation holds for each wave-length present in the radiation used. From the absorption curves, given in our previous paper [1929], we can deduce, however, that for our purpose as an approximation, the average of ϵ_{erg} may be put equal to the average of ϵ_{vit} in the range of wave-lengths under consideration. Our equation then becomes:

$$I_{erg}/I_{vit} = C_{erg}/C_{vit}.$$

Since now

$$I_{total} = I_{erg} + I_{vit}$$

we have all the necessary data to calculate the rate of disappearance of the ergosterol:

$$-(d\,C_{erg})/dt = k.I_{erg}, \text{ in which } I_{erg} = C_{erg}/(C_{erg} + C_{vit})\,.\,I_{total},$$

hence $-(d\,C_{erg})/dt = k.I_{total}.C_{erg}/C_{0\,erg}$[1] or $-(d\,C_{erg})/dt = k'\,C_{erg}$.

After integration, this equation leads to

$$\log.C_{erg}/C_{0\,erg} = k''.t, \text{ where } k'' = 2{\cdot}303\,k'.$$

We can now put this equation to the test with the data from Table I: this is done in Table II, where k'' is calculated for the two longer series of

Table II.

1	2	3	4	5	6
t	C_E/C_{E_0}	k''	t	C_E/C_{E_0}	k''
0	1·000	—	0	1·000	—
1	0·939	0·027[5]	1	0·940	0·027
2	0·907	0·021	2	0·901	0·023
4	0·796	0·025	4	0·786	0·026
6	0·709	0·025	6	0·720	0·024
8½	0·602	0·026	9	0·622	0·023
			13	0·529	0·021

measurements. From an inspection of the figures in the third and sixth columns of this table it can be concluded that the calculated factor k'' is remarkably constant in each series of experiments. This proves that our assumptions must have been fairly correct.

(c) *Crystalline preparation.*

There is still another indication for the purity of the preparations of the reaction-product obtained in this way, that is, the remarkable ease with which it is obtained in the crystalline state when the excess of unchanged ergosterol is removed by recrystallisation from ether and alcohol *in vacuo*. The procedure (including the precipitation of the residual ergosterol with digitonin) may be described somewhat more in detail.

An ethereal solution of ergosterol, *e.g.* 5 g. in 600 cc. is irradiated in the quartz apparatus during some hours, till a proper degree (*e.g.* 25 %) of transformation, as measured by the rotation, has been reached. The solution is

[1] In agreement with the assumption that only one substance is formed from the ergosterol, $C_{erg} + C_{vit} = C_{0\,erg}$, the ergosterol concentration at $t = 0$.

then transferred into another vessel, from which part of the ether is distilled. A large part of the unchanged ergosterol is then removed by cooling on ice and decanting the supernatant liquid into another vessel through a sintered glass-filter. The ether is then distilled off and replaced by about 75 cc. alcohol. By shaking vigorously the reaction-product is extracted from the crystalline mass along with only about 0·15 g. of ergosterol; then the solution is filtered into a new vessel, containing an alcoholic solution of digitonin, which precipitates the rest of the ergosterol. After filtering the liquid into a new vessel the alcohol is evaporated, and the reaction-product is extracted with ether. The ethereal solution again being filtered into another vessel the ether is distilled off; the ergosterol-free reaction-product is then obtained as a white crystalline mass, consisting of rosettes of fine needles. These crystals are very soluble in all ordinary organic solvents, but can be crystallised from strongly cooled alcohol or from diluted methyl or ethyl alcohol. Fig. 3 shows the crystals as they are obtained by evaporation of an ethereal solution.

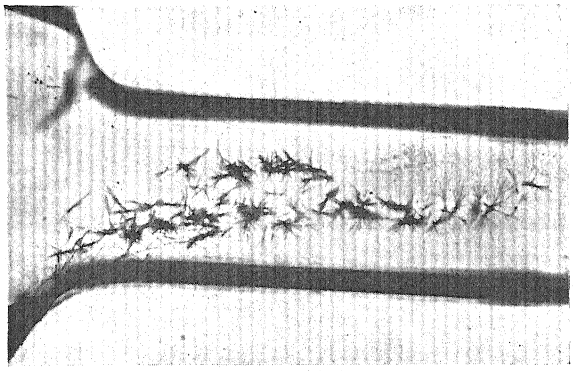


Fig. 3.

There is, however, one very interesting complication concerning this crystalline preparation, *viz.* the specific rotation is not 67°, as would be expected from the above measurements of the optical rotation of the primary reaction-product. In various experiments we have always obtained preparations with a rotation more or less exceeding the theoretical value. The longer the time between irradiation and final measurement of the rotation, and the higher the temperature used in the series of operations of recrystallisation and precipitation with digitonin or evaporation of the solvent, the higher was the value found for the rotation. For example, in one instance we found $[\alpha]_D = +94°$, in an experiment in which 20 days elapsed between irradiation and the final measurement, whilst the temperature had several times been as high as 50°. In another experiment, in which all operations had been performed as expeditiously as possible, and in which the temperature was kept as low as possible, the rotation was found to be + 68·1°.

This increase of the rotation even takes place when the solution in ether

is preserved on ice, as is shown in Table III, giving in 14 days an increase of about 7 %.

Table III.

Time	Rotation
0 days	+68·1°
3 ,,	+69·7
5 ,,	+71·1
14 ,,	+72·6

This shows that the crystalline substance is very unstable even in absence of oxygen and at a low temperature when it is dissolved in ether.

From the foregoing it follows that, though some of the physical properties of the pure vitamin can be derived comparatively easily from the properties of mixtures of vitamin D and ergosterol, other properties, for the measurement of which the vitamin D must be separated from the ergosterol, we cannot for the moment give with so much certainty.

We may, however, give some properties of one of our preparations from which the ergosterol had been removed as far as possible by means of several recrystallisations from alcohol.

Melting-point (in sealed evacuated tube) 115°–117°.

The ergosterol content of the preparation proved to be 3·7 %.

Specific rotation (corrected for ergosterol content): $[\alpha]_D = +100°$ (ether).

Analysis: H, 11·30 %, C, 85·4 %; calculated for $C_{27}H_{42}O$: H, 11·07 %, C, 84·8 %.

From the results of the analysis it may be concluded, that, during the very short time the preparation has been in contact with the air, no appreciable oxidation has taken place.

The crystals seem to be only very slightly hygroscopic, but in contact with the air gain rapidly in weight by oxidation, as is shown by the following experiment:

43·6 mg. were taken;

after 1 hour the weight was 43·7 mg.: increase 0·2 %.
,, 42 hours ,, 45·5 mg.: increase 4·4 %.
,, 66 ,, ,, 45·9 mg.: increase 5·3 %.
,, 114 ,, ,, 46·4 mg.: increase 6·4 %.

In the meantime the colour had changed from pure white to bright yellow.

That this gain in weight is not a result of absorption of water follows from the fact that it is not reversed in a high vacuum.

Even when the preparation is kept in a thoroughly evacuated desiccator and brought into contact with air only during some brief interval necessary for taking out parts of it, it acquires a slight yellow tinge within a week and becomes oxidised.

Analysis: crystals, kept *in vacuo* for 1 week: H, 10·98 %, C, 83·5 %. Crystals kept in air for 114 hours: H, 10·37 %, C, 80·1 %; calculated: H, 11·07 %, C, 84·8 %.

This proves that the crystals are very susceptible to oxidation.

The absorption spectrum of the vitamin D after preservation is not the same as that of the pure vitamin D; as we have not yet found a definite form of absorption spectrum that is common to all such preparations, we will not for the present go further into this question.

In our former paper [1929] we mentioned the fact that vitamin D can be obtained in a crystalline state. These crystals were obtained by rapidly evaporating and thus cooling an ethereal solution; they showed a melting-point below 0°. The same phenomenon can be observed if an ethereal solution of the crystalline preparation described in this paper is treated in the same way. Thus it may be concluded that the crystals mentioned in the former paper were of the same substance as the present ones, the difference in melting-point being caused by ether of crystallisation.

Discussion.

(*a*) *Rotation.* The data which are found in the literature concerning the rotation of reaction-products of ergosterol show a wide variation. Whereas the earlier work of Windaus *et al.* [1929] and of Castille and Ruppol [1929] seems to indicate that the photochemical reaction-products show a relatively small rotation, Lahousse and Gonnard [1929] find for their most potent products a value for $[\alpha]_{578m\mu}$ of + 25° (alcohol).

In a recent paper, Windaus [1930] gives the rotation of a large number of ergosterol-free reaction-products; he states that his most potent samples prepared by irradiation with the Mg-spark show a low negative rotation; a high positive rotation was found for samples prepared with long-wave irradiation (Hg-arc and Uviol-glass filter), and for aged, or heated and over-irradiated samples. The dextrorotatory, long-wave irradiation products, which we would expect to be most similar to our preparations, proved to be less active than those prepared by irradiation with the Mg-spark. The difference between the results of Windaus and our own may be due to the fact that his preparations were mostly long-time irradiation-products (*ca.* 80 % of ergosterol transformed) whereas in our experiments the degree of transformation did not exceed 50 %.

(*b*) *The crystals.* Besides the crystals mentioned in our former publication, crystalline reaction-products from irradiated ergosterol have been described by Windaus [1930; Windaus *et al.* 1930] and by Askew *et al.* [1930, 1, 2]. Whereas the crystals described by Windaus are produced by over-irradiation and consequently do not show antirachitic activity, the crystalline preparation of Askew *et al.* shows a very intense activity and therefore consists at least partly of vitamin D. The melting point as well as the form of the crystals and the absorption spectrum suggest a similarity between these crystals and ours.

Since writing the above, we have had the privilege of discussing the subject with the English authors, who kindly informed us that their further purified crystalline preparation shows a much higher dextrorotation than ours; this

proves that the substance must be different. As to the nature of our crystals, we already pointed out that they probably do not consist of pure vitamin D, since the rotation of the crystalline preparation does not as a rule tally with the calculated rotation for pure vitamin D.

The way in which Askew *et al.* have obtained their crystalline preparation, *viz.* by distillation *in vacuo* of the crude reaction-product, implies the necessity of heating the vitamin D to temperatures above 100°; therefore in these experiments too the vitamin D is likely to undergo a change of the same nature as that occurring in our method of preparation.

The instability of vitamin D on storage or on heating has been described by Windaus [1930; Windaus and Auhagen, 1931] and by Askew *et al.* [1930, 1]. Though in some respects our results are not very different from those of these authors, there still exist a good many points in which they differ. Since, however, the experiments have been performed with reaction-products which may show a good deal of dissimilarity, the difference in the results does not at the present time provide a useful starting-point for a discussion. We wish to emphasise however one point of difference, *viz.* that, contrary to the experience of the other authors, we have never found an increase in absorption if the ergosterol-free reaction-product was irradiated with short-wave ultra-violet rays [Reerink and v. Wijk, 1931].

Summary.

Experiments are described in which the change of the rotation of ergosterol solutions on irradiation with long-wave ultra-violet rays is measured.

If the necessary precautions are taken, this change is very simple, the rotation showing a linear dependence on the degree of transformation of the ergosterol. These experiments provide further proof for the hypothesis, put forward in our previous publication, *viz.* that under definite conditions long-wave irradiation of ergosterol gives rise to one reaction-product only, which, on account of its intense antirachitic activity, must be vitamin D.

From these experiments the rotation of vitamin D can be calculated to be

$$[\alpha]_D = + 66{\cdot}7° \pm 2{\cdot}7° \text{ (in ether).}$$

From correctly irradiated solutions, crystalline ergosterol-free preparations of vitamin D may easily be produced by recrystallisation and treatment with digitonin *in vacuo*. The properties of these preparations vary with the time and temperature used during the performance of the necessary operations, as a result of the instability of the vitamin D.

In conclusion we wish to express our thanks to Mr W. Breen and Mr J. Freen for their valuable assistance in the performance of the experiments.

REFERENCES.

Askew, Bourdillon, Bruce, Jenkins and Webster (1930, 1). *Proc. Roy. Soc. Lond.* B **107**, 77.
—— —— —— —— —— (1930, 2). *Proc. Roy. Soc. Lond.* B **107**, 91.
Bills and Honeywell (1928). *J. Biol. Chem.* **80**, 15.
Castille and Ruppol (1929). *Bull. Acad. Roy. Med. Belg.* 799.
Everse and v. Niekerk (1931, 1). *Ned. Tijdschr. Geneesk.* **75**, 1101.
—— —— (1931, 2). *Strahlentherapie*, **40**, 728.
Lahousse and Gonnard (1929). *J. Soc. Franç. Physique*, **280**, 114.
Reerink and v. Wijk (1929). *Biochem. J.* **23**, 1294.
—— —— (1931). *Strahlentherapie*, **40**, 728.
Windaus, (1930). *Nachr. ges. Wiss.* (Göttingen), 36.
—— and Auhagen (1931). *Z. physiol. Chem.* **196**, 108.
—— Gaede, Köser and Stein (1930). *Liebig's Ann.* **483**, 17.
—— Westphal, v. Werder and Rugh (1929). *Nachr. ges. Wiss.* (Göttingen), 45.

CXII. THE OXIDATION OF HEXOSE-DIPHOSPHORIC ACID BY AN ENZYME FROM ANIMAL TISSUES.

By DOUGLAS CREESE HARRISON.

From the Department of Pharmacology, Sheffield University.

(*Received June 2nd, 1931.*)

Thunberg [1929] described the preparation from cucumber seeds of an enzyme which, in presence of methylene blue, was capable of oxidising citric acid. Of a very large number of possible substrates tested, hexosediphosphoric acid was the only substance other than citric acid which in dilute solution was oxidised by the enzyme preparation. A short time earlier, Bernheim [1928] described the preparation of an enzyme from liver which, in the presence of methylene blue, brought about the oxidation of citric acid. It seemed therefore of interest to see whether this enzyme preparation from liver could, like that from cucumber seed, also oxidise hexosediphosphate. The following experiment shows that hexosediphosphoric acid is indeed oxidised by the enzyme prepared from liver.

Acetone-liver was prepared from fresh ox liver by the method described by Bernheim [1928]. 30 g. of the dry preparation were extracted with 100 cc. of distilled water in a large mortar, the mixture being ground at intervals for $2\frac{1}{2}$ hours. After straining and squeezing through muslin, the residue was again ground for a few minutes with 50 cc. of distilled water and squeezed through muslin. The combined extracts were centrifuged, and the cloudy solution obtained was saturated with ammonium sulphate and allowed to filter overnight in the ice-chest. The precipitate was dissolved in phosphate buffer, the volume being made up to 45 cc., and was brought to p_H 7·6 with dilute sodium hydroxide and centrifuged to remove insoluble protein.

The sodium hexosediphosphate solution was prepared from a pure sample of calcium hexosediphosphate (calcium fructo-furanose-1:6-diphosphate) obtained from Bayer Products, Ltd. A weighed amount was dissolved in water, treated with very slightly less than the equivalent amount of pure sodium oxalate and centrifuged. After testing to ensure freedom from oxalate, the solution was stored below 0°, fresh solutions being made up at frequent intervals.

Exp. 1. The experiment was carried out using Thunberg vacuum tubes which were evacuated at the water-pump, filled with nitrogen, re-evacuated and put into a water-bath at 37°. All experiments described in this paper were carried out at this temperature. Each tube contained 1·5 cc. enzyme, 0·5 cc. phosphate buffer p_H 7·6, and 0·25 cc. methylene blue (1 in 5000).

Substrate added	Time for decoloration of methylene blue mins.
None	45
0·0125 cc. *M*/5 Na hexosediphosphate	21
0·025 cc. ,,	20
0·0125 cc. *M*/5 Na citrate	17
0·025 cc. ,,	15

A dialysed enzyme preparation made exactly according to Bernheim's method similarly oxidised hexosediphosphate. A solution of sodium hexosediphosphate prepared by acting on the barium salt (obtained from another source) with sodium sulphate gave similar reduction times with methylene blue. By precipitating the enzyme twice with ammonium sulphate the control time is lengthened, and the acceleration due to hexosediphosphate becomes relatively much greater, as is seen in Exp. 4.

Having shown that the hexosediphosphoric enzyme is associated with the citric enzyme not only, as shown by Thunberg, when prepared from cucumber seeds, but also when prepared from liver, an experiment was carried out to determine whether the oxidation of both substrates was brought about by the same enzyme. If one of the substrates be added to the enzyme in such amount that further addition causes no increase in the rate of reduction of methylene blue, the enzyme surface may be regarded as being saturated with the substrate. The further addition of the other substrate may produce one of two effects. If the same enzyme is responsible for the oxidation of both substrates, the two substrates will compete for the active surface of the enzyme, and the time of reduction of methylene blue with the mixture will be between the reduction times of the two substrates alone. On the other hand, if the oxidation of the two substrates is brought about by two different enzymes, when both substrates are present the two reactions will be occurring simultaneously on different enzyme surfaces, and the resulting rate of reduction will be greater (*i.e.* the time of reduction of methylene blue will be less) than that with either substrate alone. The following experiment shows that in the case of citric acid and hexosediphosphoric acid the latter alternative occurs and that the two substrates are therefore oxidised by different enzymes.

Exp. 2. Each vacuum tube contained 1·5 cc. enzyme + 0·5 cc. phosphate buffer p_H 7·6 + 0·25 cc. methylene blue. Water was added to make the total volume in each tube 2·45 cc.

Substrate added	Time for decoloration of methylene blue mins.
None	39
0·05 cc. *M*/10 Na hexosediphosphate	18
0·1 cc. ,,	17½
0·05 cc. *M*/10 Na citrate	12
0·1 cc. ,,	14
0·05 cc. *M*/10 Na hexosediphosphate + 0·05 cc. *M*/10 Na citrate	8½
0·1 cc. *M*/10 Na hexosediphosphate + 0·1 cc. *M*/10 Na citrate	7

The values for the mixed substrates agree fairly closely with the values calculated on the assumption that both reactions take place independently. This suggests that neither substrate is appreciably adsorbed on the enzyme which oxidises the other substrate, since such adsorption would be expected to produce an inhibition in the rate of oxidation [Quastel and Wooldridge, 1927].

An attempt was made to prepare the enzyme from rat muscle. 8 g. of fresh rat leg muscle was finely chopped and extracted with three times its weight of distilled water for $2\frac{1}{2}$ hours. The extract was strained through muslin and precipitated by saturation with ammonium sulphate. The precipitate was pressed on filter-paper, made up to 5 cc. in phosphate buffer and brought up to p_H 7·5 with dilute NaOH.

Exp. 3. Each vacuum tube contained 1 cc. enzyme + 0·25 cc. phosphate buffer p_H 7·5 + 0·125 cc. methylene blue.

Substrate added	Time for decoloration of methylene blue mins.
(0·1 cc. water)	130
0·1 cc. *M*/25 Na hexosediphosphate	40
0·1 cc. *M*/25 Na citrate	90

An enzyme solution can thus be extracted from rat muscle showing an activity towards hexosediphosphate similar to the activity of the enzyme extracted from an equal weight of ox liver. While, however, enzyme preparations from the latter usually show an approximately equal activity towards equimolecular solutions of citrate and hexosediphosphate, the enzyme solution from rat muscle contains relatively little citric dehydrogenase. Preparations have been obtained from rat muscle showing considerable activity towards hexosediphosphate and practically no activity towards citrate. Incidentally, this affords confirmation that the activation of the two substrates is brought about by different enzymes.

A similar preparation from fresh young rabbit leg muscle showed some activity towards hexosediphosphate but hardly any towards citrate. Other tissues have not so far been tested for the presence of the enzyme.

Experiments carried out with the Barcroft differential manometer at 37° indicate that while the enzyme solution by itself shows a small residual oxygen uptake, the addition of hexosediphosphate does not produce any appreciable increase in the oxygen uptake. If, however, methylene blue be added, a definite increase in oxygen uptake is observed, as would be expected, since the leuco-methylene blue formed by reduction of methylene blue takes up oxygen spontaneously. The inability of the enzyme to bring about direct oxidation of hexosediphosphate with oxygen does not in any way indicate that such an oxidation does not occur in the body. In the intact cell, there is good reason to believe [Keilin, 1929] that through the agency of cytochrome and the intracellular oxidase, active oxygen is made available for the oxidation of substrates of such dehydrogenase systems as the present one. That such oxidations can occur is shown in the next paper [Harrison, 1931].

Various workers have shown the presence of an enzyme in tissues capable of oxidising glycerophosphoric acid, and this substance was therefore tested as being a possible substrate for the hexosediphosphate dehydrogenase.

Exp. 4. Each vacuum tube contained 1 cc. enzyme solution (twice precipitated by saturation with ammonium sulphate) in phosphate buffer at p_H 7·4 and 0·33 cc. methylene blue.

Substrate added	Time for decoloration of methylene blue mins.
0·07 cc. water	77
0·07 cc. *M*/25 Na glycerophosphate	68
0·07 cc. *M*/25 Na hexosediphosphate	17

This experiment has been repeated a number of times with similar results. The glycerophosphate appears therefore to be oxidised to a very slight extent by the enzyme preparation, but the rate of oxidation is negligible compared with that of hexosediphosphate.

Properties of the enzyme preparation.

Both the citric and hexosediphosphoric dehydrogenases are only partially precipitated by half-saturation with ammonium sulphate, though half-saturation appears to bring down a greater proportion of the hexosediphosphoric enzyme than of the citric enzyme. Thus, although a large part of the hexosediphosphoric enzyme remains in the filtrate, a preparation of this enzyme may be obtained with the citric acid enzyme partially removed, and with the additional advantage of showing only a very small residual reducing power towards methylene blue in the absence of substrate.

If the liver extract be dialysed overnight in the ice-chest in a collodion sac, then acidified to p_H 5·7 and centrifuged before precipitation with ammonium sulphate, a perfectly clear solution of the enzyme can be obtained. This clear solution retains its activity even after filtration through kieselguhr or through a porcelain candle (Pasteur-Chamberland L 5).

While the hexosediphosphate enzyme present in the dry acetone-liver preparation is fairly stable if kept *in vacuo*, the solution of the enzyme is rather unstable and loses some of its activity even on standing overnight in the ice-chest. A solution of the enzyme in water at p_H 7·2 lost about one-third of its activity after being heated to 45° for half an hour. A neutral solution of the preparation brought to about p_H 4 with HCl, allowed to stand a few minutes and again neutralised, was not appreciably reduced in activity. After some hours at this p_H, however, the activity towards both citrate and hexosediphosphate is completely lost. The oxidation of hexosediphosphate falls off rapidly on the acid side of neutrality. The activity in phosphate buffer is much less at p_H 6 than at p_H 7.· It is somewhat greater at p_H 8 than at p_H 7. Owing, however, to the loss of ammonia from the alkaline solutions with consequent change of p_H during evacuation of the tubes, and owing to the fact that the

reaction is strongly inhibited by borate and phthalate buffers, a satisfactory p_H-activity curve has not so far been obtained.

The optimum substrate concentration for the hexosediphosphate dehydrogenase at p_H 7·6 appears to be about $M/450$ and is of the same order as that for the citric dehydrogenase.

Since citric acid and hexosediphosphate are oxidised by different enzymes, it seems remarkable that these two enzymes should occur together relatively free from other enzymes when prepared from sources so different as cucumber seed and ox liver. The two enzymes show a considerable similarity in their physical properties as is shown by the lack of success of a number of attempts to separate the two by warming and by treatment with various adsorbents. It seems likely that the centres responsible for the two activations are closely associated on the same large protein molecule, and that this protein largely determines the physical properties of the enzymes.

No evidence has so far been obtained as to the nature of the product or products of this oxidation of hexosediphosphoric acid, and one can only speculate on the function of the enzyme in the body. Meyerhof [1930] has shown that during glycolysis in muscle, hexosediphosphate is built up, and in view of the high concentration of the hexosediphosphate dehydrogenase which I have shown to be present in muscle, it seems possible that the oxidation of hexosediphosphate takes place side by side with that of lactic acid during the recovery period in muscle.

SUMMARY.

1. An enzyme (dehydrogenase) has been obtained from liver and muscle capable of oxidising hexosediphosphoric acid in presence of methylene blue.

2. In spite of the association of this enzyme with a citric acid dehydrogenase both when prepared from cucumber seed and from ox liver, the two enzymes are shown nevertheless to be distinct.

The author wishes to express his thanks to Prof. E. Mellanby for his interest in this work.

Part of the expense was defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

Bernheim (1928). *Biochem. J.* **22**, 1178.
Harrison (1931). *Biochem. J.* **25**, 1016.
Keilin (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
Meyerhof (1930). Die chemischen Vorgänge im Muskel, p. 157. (Berlin, Springer.)
Quastel and Wooldridge (1927). *Biochem. J.* **21**, 1240.
Thunberg (1929). *Amer. J. Physiol.* **90**, 540.

CXIII. GLUCOSE DEHYDROGENASE: A NEW OXIDISING ENZYME FROM ANIMAL TISSUES.

By DOUGLAS CREESE HARRISON.

From the Department of Pharmacology, Sheffield University.

(*Received June 2nd, 1931.*)

The preparation and properties of an enzyme which oxidises hexosediphosphate have already been described [Harrison, 1931]. During the course of this work, experiments were carried out to see whether the oxidation of hexosediphosphate was affected by the addition of other substances. Substances similar in structure to hexosediphosphate might, from the work of Quastel and Wooldridge [1928], be expected to be adsorbed at the same enzyme surface and so inhibit the oxidation. One of the substances tested was glucose, and it was surprising to find that this substance produced not an inhibition but a marked acceleration. Further investigation showed that there is present in the hexosediphosphate enzyme preparation another enzyme capable, in the presence of a suitable hydrogen acceptor, of bringing about the oxidation of glucose. Since an enzyme which oxidises glucose has not apparently been hitherto obtained from animal tissues, it seemed desirable to study its preparation and properties in some detail. It has been found possible to obtain perfectly clear solutions of the enzyme capable of being filtered through a porcelain filter without loss of activity.

Experimental.

Preparation of the enzyme.

Acetone-liver was prepared by a modification of the method described by Bernheim [1928]. About 1½ lbs. of fresh ox liver (obtained a few hours after the animal was killed) were weighed after peeling off the surrounding membrane, washed for about a minute under the cold tap, squeezed and minced twice through an ordinary fine mincer. The pulp was well stirred for about a minute with pure acetone (B.P.), using 900 cc. to 675 g. of liver, and filtered on a large Büchner funnel. The acetone treatment was repeated twice more, using half the above quantity of acetone for each extraction. After partially drying on the funnel for a short time, the product was spread out in two large desiccators over sulphuric acid and evacuated at the water-pump for several hours until most of the acetone was removed. The preparation is fairly stable if kept evacuated in the desiccator. In order to extract the enzyme, 30 g. of the

dry acetone-liver were put into a mortar with 120 cc. of distilled water and ground at intervals for 2 to 3 hours. The mixture was squeezed through coarse muslin, the residue ground up with 60 cc. of distilled water, squeezed through muslin, and the combined extracts were centrifuged for a short time. The opaque liquid was poured off (through muslin to remove the fatty layer which sometimes forms), put into thin collodion sacs, and dialysed overnight in the ice-chest against 2 litres of distilled water. The solution was then brought to about p_H 5·7 to bromocresol purple by the addition of about one drop of *N* HCl and the heavy protein precipitate centrifuged off. The clear or slightly opaque pigmented solution was brought approximately to neutrality by the addition of a few drops of *N* NaOH and was saturated with pure ammonium sulphate. After carefully pouring off from the excess of ammonium sulphate, the precipitate was filtered in the ice-chest on a starch-free filter-paper (Whatman, No. 40), partially dried by transferring to an absorbent porous tile, and finally removed from the plate and dried *in vacuo* over sulphuric acid. The enzyme, which is fairly stable in the dry form, was dissolved in water or phosphate buffer, made up to the required volume (30 cc. or 45 cc. are convenient volumes for methylene blue experiments) and brought to p_H 7·4 with *N* NaOH. This solution which is pigmented, but as a rule perfectly clear, was used for most of the experiments described in this paper. If the enzyme is required free from ammonium sulphate, the dry preparation is put into a wet collodion sac and dialysed against frequent changes of distilled water in the ice-chest until practically free from ammonium sulphate, after which it is centrifuged, a considerable amount of protein being thereby removed. The solution may then be concentrated by bringing to about p_H 7·8 with NaOH and distilling *in vacuo* in a large flask, keeping the temperature of the liquid below 25° and cooling the receiver in ice. The flask is heated on a water-bath at 30–35°. Frothing is sometimes troublesome at the beginning of the distillation, and it is advisable to allow a very slow stream of air (one bubble every few seconds) to bubble through a wide capillary under the surface of the liquid. If dialysis has been complete, the solution remains alkaline at the end of the distillation, and there is practically no destruction of the enzyme. The enzyme does not keep well in solution and loses some of its activity even on standing overnight in the ice-chest.

In preparing the enzyme, the following points should be noted. The temperature during the acetone treatment should not be much above 20°, and in hot weather, the acetone and the liver should be first cooled in the ice-chest. The acetone treatment should be carried out as rapidly as possible, and prolonged evacuation of the desiccators is desirable in order to remove the acetone as completely as possible. The collodion sacs used for dialysis should be kept sterile and should be renewed after using about three times, as they tend to become impermeable. In drying the enzyme, the thick absorbent porous plate is much preferable to the ordinary unglazed dinner plate. (The "Owens College" pattern from Griffin and Tatlock, Ltd., was found satisfactory.) The use of

ammonium sulphate in fairly large crystals facilitates decantation of the precipitated enzyme from the excess of ammonium sulphate. Starch-free filter-paper should always be used (except in preparing the acetone-liver) since the liver extract may break down starch to glucose.

The activity of the enzyme prepared from different samples of liver varies considerably, and occasionally an almost inactive preparation is obtained. The samples of liver which are soft and mince to a dark almost liquid pulp usually give the most active enzyme preparations. The enzyme has been prepared from the liver of the ox, sheep, cat and dog. Other tissues have not yet been examined.

Anaerobic oxidation of glucose.

The enzyme solution by itself usually shows a slight reducing power towards methylene blue varying considerably with different preparations. On adding glucose, the rate of reduction of methylene blue is greatly increased, as is shown in the following typical experiment. The methylene blue experiments were carried out at 37° in Thunberg vacuum tubes as previously described [Harrison, 1931]. 1 cc. of enzyme solution equivalent to 1 g. of acetone-liver was used in each tube together with 0·5 cc. phosphate buffer p_H 7·4 and 0·5 cc. methylene blue (1 : 5000). The glucose used was pure dextrose (A.R.), from British Drug Houses. The solutions of glucose were stored below 0° and fresh solutions were made up at frequent intervals.

Substrate added	Time of decoloration of methylene blue
None	28 mins.
0·05 cc. 2 *M* glucose	5 mins.

That the acceleration is due to glucose itself and not to an impurity is clear from the fact that different samples of pure glucose from different sources in suboptimal concentrations gave, with the same enzyme preparation, identical reduction times.

Pure fructose is not oxidised by the enzyme, and in the same concentration as glucose in the above experiment retards the residual reducing power of the enzyme preparation itself. Galactose and arabinose showed no appreciable reduction of methylene blue except in high concentrations, and it is not unlikely that the slow reduction which then occurred was due to traces of glucose present as an impurity in the sugars.

The effect of increasing the glucose concentration is shown in Fig. 1. The experiment was carried out at 37° using 1·0 cc. ammonium sulphate-free enzyme (equivalent to 1 g. acetone-liver) and 0·5 cc. methylene blue. The p_H was 7·6, and water was added where necessary to bring the total volume to 2·0 cc.

The optimum concentration of glucose is seen to be 0·25 *M* and in concentrations greater than this the velocity falls off slowly. The substrate concentration giving half the maximum velocity is about 0·07 *M* which is an exceptionally high value for an oxidising enzyme.

Lactic, citric and hexosediphosphoric acids are oxidised, though the optimum rates of oxidation with these substances are considerably smaller than that of glucose. Since, however, lactic acid is oxidised by the preparation, it was possible that the oxidation of glucose might not be a direct one, but that the glucose might first be glycolysed to lactic acid and the lactic acid then oxidised. That such is not the case is shown by the following experiment.

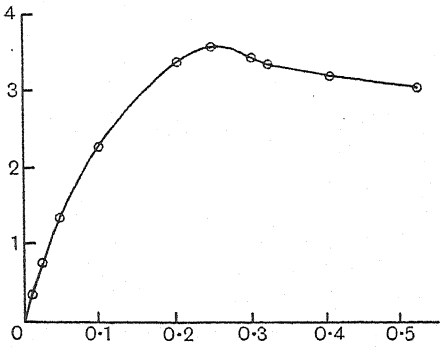


Fig. 1. Rate of glucose oxidation as a function of substrate concentration. Abscissa: molar concentration of glucose. Ordinate: velocity $= 100/T$, where T = methylene blue reduction time in minutes.

Four small flasks A, A', B, B', each contained 10 cc. of enzyme solution. To duplicates B and B' were added 3·0 cc. M glucose, while the duplicates A and A' received 3 cc. water. The flasks were then filled with nitrogen, tightly stoppered and incubated at 37° for $2\frac{1}{2}$ hours. 5 cc. of 20 % trichloroacetic acid were then added to each flask, the solutions were filtered, glucose removed by the copper-lime method and lactic acid was estimated on an aliquot portion of each solution by the method of Friedemann, Cotonio and Shaffer [1927].

Flask	Mg. lactic acid
A	0·94
	= 0·90
A'	0·87
B	0·72
	= 0·73
B'	0·74

It is clear that there is no formation of lactic acid from the added glucose. Methylene blue experiments carried out at the same time with the same enzyme gave the following results. Each tube contained 1 cc. enzyme + 0·25 cc. methylene blue (1 in 5000) together with sufficient water to make the volume 1·55 cc.

Substrate added	Time for decoloration of methylene blue mins.
None	50
0·3 cc. M/1 glucose	5
0·1 cc. M/10 lactate	25
0·05 cc. M/10 hexosediphosphate	19
0·05 cc. M/10 hexosediphosphate + 0·1 cc. M/10 lactate	14
0·05 cc. M/50 hexosediphosphate + 0·1 cc. M/50 lactate	27

This shows that an amount of lactic acid much greater than the experimental error in the estimation of lactic acid produces only a relatively small acceleration in the rate of reduction of methylene blue.

The experiments on the mixture of hexosediphosphate and lactate were carried out, since the glucose enzyme preparation also contains an enzyme capable of oxidising hexosediphosphate, and Meyerhof and Lohmann [1927] have given evidence that in the lactic acid production in muscle extract with glucose, one molecule of hexosediphosphoric acid is formed with every two molecules of lactic acid. Mixtures of hexosediphosphate and lactate in the ratio of one molecule of the former to two of the latter in concentrations of $M/50$ and $M/10$ were found to be oxidised much more slowly than glucose. These results, coupled with the fact that no lactic acid could be detected on incubating the enzyme with glucose anaerobically, show that the oxidation of glucose by the enzyme is not due to the formation and oxidation of lactic acid either alone or with hexosediphosphoric acid. Indirect evidence in favour of this conclusion is furnished by the fact that neither sodium fluoride nor iodoacetic acid, both of which inhibit lactic acid formation in muscle, produces any inhibition in the rate of reduction of methylene blue by glucose *plus* the enzyme. (The experiments were carried out with $N/100$ sodium fluoride and with $N/1000$ sodium iodoacetate, both at p_H 7·6.)

A mixture of glucose and hexosediphosphate, the latter being above its optimum concentration, reduced methylene blue more quickly than either of the substrates in the same concentration by themselves. By the line of reasoning previously employed in proving the non-identity of the citric and hexosediphosphate enzymes [Harrison, 1931], this indicates that the oxidation of glucose and hexosediphosphate is brought about by two different enzymes, rather than by the same enzyme acting on two different substrates.

Properties of the enzyme.

In studying the effect of p_H on the activity of the enzyme, the dialysed enzyme free from ammonium sulphate was used, and glucose was added at its optimum concentration. No buffer was added, since the protein in the solution possesses a large buffering power. It was found that at p_H 6–8, the activity at 37° remained approximately constant. At p_H 9–9·5 the activity was apparently greater, but this was found to be due, at least in part, to the increased reducing power towards methylene blue of the enzyme solution alone. In still more alkaline solutions, glucose by itself slowly reduces methylene blue. In phosphate buffer of p_H 8·0, glucose by itself showed no reduction of methylene blue even after 10 hours at 37°.

The glucose dehydrogenase is completely precipitated, together with all the pigments, by saturation with ammonium sulphate. Attempts to precipitate the enzyme without precipitating the pigments were not very successful, as the enzyme is only partially precipitated even by 75 % saturation with ammonium sulphate. Owing to the difficulty of obtaining the enzyme free from ammonium

sulphate, a number of other possible precipitants were tried without success. The precipitates obtained with 50%, 75%, and 90% acetone showed little or no activity even on carrying out the precipitation with ice-cold acetone. Similarly, the product obtained by precipitation with 90% alcohol keeping the mixture below 0° was inactive.

The enzyme may be filtered without loss through kieselguhr, whereby some pigment is removed and freedom from insoluble cell material is ensured. The enzyme may also be filtered through a fine porcelain candle (Pasteur-Chamberland L 5) without loss of activity. With the concentrated enzyme, filtration may be slow unless the solution is first filtered through kieselguhr. The dilute solution obtained after dialysing and centrifuging the extract of acetone-liver, however, filters through the candle readily and can then be saturated with ammonium sulphate yielding a preparation free from cell debris and giving a perfectly clear solution in water.

A number of attempts were made to purify and concentrate the enzyme by adsorption methods. Most of these experiments were carried out with the dialysate from acetone-sheep liver before precipitation with ammonium sulphate. It was found difficult to standardise the conditions since different enzyme preparations contained different amounts of protein. Treatment of 20 cc. of dialysate (p_H 5·7) five times with 0·5 g. of charcoal (Merck's Medicinal), stirring for a minute and filtering under suction each time, followed by precipitation with ammonium sulphate usually gave an enzyme containing much less pigment and with its activity unimpaired. By continuing the charcoal treatment, more pigment is removed, but the enzyme is also adsorbed from the solution. The enzyme does not appear to be adsorbed from the untreated dialysate by kaolin or kieselguhr either in acid or alkaline solution. If, however, part of the protein be first removed by treating about five times with charcoal, the enzyme can be adsorbed from acid solution by kaolin. Thus, by stirring 10 cc. of the charcoal-treated dialysate three times with 0·5 g. kaolin at p_H 4·5 or 5·5, about two-thirds of the enzyme was removed from the solution. Treatment of the kaolin with $N/10$ K_2HPO_4 followed by precipitation of the eluted enzyme with ammonium sulphate gave a solution with some activity towards glucose, though much of the enzyme was lost.

The effect of temperature on the destruction of the enzyme depends on the nature of the enzyme preparation. For example, the presence of ammonium sulphate appears to protect the enzyme. A solution of the ammonium sulphate-precipitated enzyme, after heating at 52° in nitrogen at p_H 7·3 for 5 minutes and centrifuging off the precipitate of inactive protein, was found to have lost little of its activity. On the other hand, the enzyme dialysate before precipitation with ammonium sulphate, warmed under the same conditions, lost nearly half of its activity. The ammonium sulphate-precipitated enzyme solution at p_H 7·3 lost about one-third of its activity after heating at 45° for half an hour. The enzyme is rapidly destroyed at 60° even in presence of ammonium sulphate.

The reduction of methylene blue by glucose in presence of the enzyme was not inhibited by the presence of neutralised $M/300$ KCN. The addition of 0·048 mg. (= 1 unit) of insulin hydrochloride (British Drug Houses) in a total volume of 2·1 cc. produced no effect on the rate of reduction of methylene blue. The enzyme is not destroyed by toluene, and several preparations were made by grinding liver with toluene, extracting with water and precipitating the enzyme with ammonium sulphate.

The aerobic oxidation of glucose.

A large number of experiments were carried out in the Barcroft apparatus to determine whether the system glucose-oxidising enzyme *plus* glucose was capable of taking up oxygen directly. The results have been rather inconclusive, for while with a considerable number of preparations of the enzyme, the oxygen uptake has been somewhat greater in presence of glucose than in its absence, this extra uptake being unaffected by KCN, yet with many other preparations of the enzyme, no oxygen uptake with glucose has been obtained. Most of these experiments were carried out with the ammonium sulphate-containing preparation, but in those experiments in which the dialysed ammonium sulphate-free enzyme was used, it is noteworthy that in no case was an oxygen uptake observed. It was also noted that, with most enzyme preparations, at the end of the experiment the solution containing glucose was definitely more acid than at the beginning, and more acid than the control without glucose. These observations suggested the following explanation as possibly accounting for the apparent oxygen uptake observed in some cases. The glucose, while not being directly oxidised by oxygen, may possibly be able to use as a hydrogen acceptor some reducible substance in the enzyme preparation, for example, a reducible pigment. The formation of an acid oxidation product of glucose oxidised in this manner would account for the development of acidity in the solution. (Formation of lactic acid by glycolysis has already been excluded.) At the beginning of all the experiments, the solution was definitely alkaline (p_H 7·4 or 7·6). When the ammonium sulphate-containing preparation was used, free ammonia would thus be present in the gas phase. During the course of the experiment, the development of acidity in the solution would cause the absorption of this ammonia gas, which would be read on the manometer as an apparent uptake of oxygen.

When methylene blue was added to the solution in the Barcroft apparatus, an oxygen uptake with glucose was always observed, the methylene blue being alternately reduced by glucose and oxidised by molecular oxygen. It seemed therefore that in the oxidation of glucose with glucose dehydrogenase, molecular oxygen cannot act as a hydrogen acceptor. It was thus of interest to see whether the aerobic oxidation could be brought about in presence of active oxygen.

Oxidation of glucose in presence of the cytochrome and indophenol oxidase system.

Keilin [1929] has shown that cytochrome is oxidised by oxygen activated by indophenol oxidase and that it is reduced by dehydrogenase systems. Cytochrome is believed to act as a carrier between the oxygen activators and the hydrogen activators of the cell. Keilin [1930] has shown that the oxidation of cysteine is greatly accelerated by indophenol oxidase in presence of cytochrome, the latter acting as a carrier between the activated oxygen and the sulphydryl group. The oxidation of an actual dehydrogenase system by cytochrome *plus* indophenol oxidase has not hitherto been experimentally brought about. One of the difficulties is that the indophenol oxidase which is prepared from heart muscle, itself contains certain dehydrogenases. Preliminary experiments with methylene blue having shown that the indophenol oxidase prepared from heart muscle does not contain any glucose dehydrogenase, an attempt was made to build up the complete oxidising system using glucose dehydrogenase, that is, to find whether glucose *plus* glucose dehydrogenase would take up oxygen in presence of cytochrome *plus* indophenol oxidase. The following experiments and the curves in Fig. 2 show that in presence of the complete system there is a rapid oxygen uptake.

A fresh solution of cytochrome *c* was prepared from 2 lbs. of Delft yeast by the first method of Keilin [1930]. The precipitate obtained with SO_2 was well washed, suspended in water, brought to p_H 7·4 with NaOH and made up to 7 cc. Indophenol oxidase was prepared by a method similar to that of Keilin by grinding fresh sheep's heart muscle with fine washed sand, using 50 cc. of dry sand to 20 g. of muscle. The very fine suspension in water was centrifuged, washed twice with distilled water and evenly suspended in phosphate buffer, 14 g. of wet pulp being made up to a volume of 50 cc. and brought to p_H 7·4. This very strong suspension was used, since during the experiment the particles become aggregated on the sides of the vessel, the enzyme surface being thereby much reduced. The glucose dehydrogenase solution was an ammonium sulphate-free preparation prepared as already described. At 37°, 0·4 cc. (equivalent to 1 g. of dry liver) + 0·6 cc. p_H 7·4 buffer reduced 0·5 cc. methylene blue in 10 minutes in presence of 0·025 cc. 2*M* glucose and in 30 minutes without glucose. Four Barcroft differential manometers were used, the right-hand bottle of each receiving 1·7 cc. of glucose dehydrogenase. The first received in addition 0·8 cc. p_H 7·4 phosphate buffer; the second, 0·8 cc. buffer and 0·2 cc. 2*M* glucose; the third, 0·8 cc. indophenol oxidase and 0·3 cc. cytochrome; the fourth, 0·8 cc. oxidase and 0·3 cc. cytochrome and 0·2 cc. 2*M* glucose. Water was added to make the volume in each bottle up to 3·0 cc. The left-hand bottles each contained 3·0 cc. buffer. The side-tube of all bottles contained 5% NaOH soaked on a roll of filter-paper to absorb CO_2. The apparatus were shaken in a water-bath at 37°.

The oxygen uptakes after 110 minutes were as follows:

	mm.³	Final p_H
1. Dehydrogenase	83	7·2
2. Dehydrogenase + glucose	85	7·2
3. Dehydrogenase + cytochrome + oxidase	189	7·0
4. Dehydrogenase + cytochrome + oxidase + glucose	352	6·7

The course of the oxygen uptake is shown in the corresponding curves in Fig. 2.

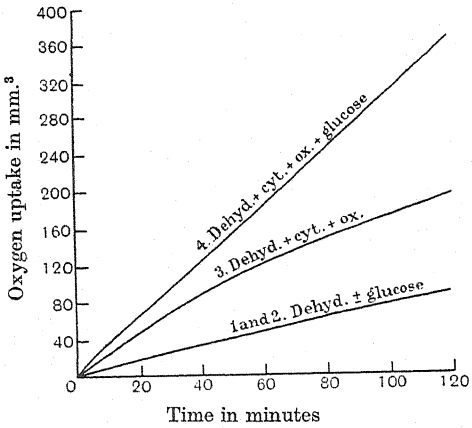


Fig. 2. Oxygen uptake of glucose in presence of dehydrogenase, cytochrome and indophenol oxidase.

It is clear that while no oxidation of glucose was brought about by the dehydrogenase itself, a large oxidation of glucose was induced by the dehydrogenase in presence of the oxygen-activating system, cytochrome and indophenol oxidase. Incidentally, the greater uptake in 3 compared with 1 shows that the cytochrome-oxidase system also increased the oxygen uptake of the residual reducing systems in the dehydrogenase preparation.

This experiment has been repeated a number of times with different preparations of the enzymes, and similar results have always been obtained. In spite of the highly buffered solutions, the p_H at the end of the experiment was always definitely lower in the solution in which oxidation of glucose had taken place. It appears that the oxidation product of glucose is an acid.

The following experiment was carried out to show that the complete system, glucose dehydrogenase, cytochrome and indophenol oxidase, is necessary in order to bring about the oxidation of glucose; *i.e.* that neither cytochrome nor the oxidase alone will induce the oxidation. The cytochrome, oxidase and dehydrogenase solutions were the same as used in the previous experiment, stored overnight at 0°. Five Barcroft apparatus were used, the solutions in the two bottles of any one apparatus being the same except that each right-hand bottle contained glucose while each left-hand bottle contained an equal volume of water. The bottles of No. 1 each contained 1·7 cc. glucose dehydrogenase and 0·8 cc. p_H 7·4 phosphate buffer; No. 2 contained 1·7 cc. dehydro-

genase and 0·8 cc. indophenol oxidase; No. 3 contained 1·7 cc. dehydrogenase, 0·3 cc. cytochrome and 0·8 cc. buffer; No. 4 contained 1·7 cc. dehydrogenase, 0·3 cc. cytochrome and 0·8 cc. oxidase; No. 5 contained 0·3 cc. cytochrome, 0·8 cc. oxidase but no dehydrogenase. After adding 0·2 cc. 2*M* glucose to each of the right-hand bottles, sufficient water was added to make the volume in each bottle equal to 3·0 cc.

The oxygen uptake after 170 minutes at 37° was as follows:

	mm.³
1. Dehydrogenase + glucose	19
2. Dehydrogenase + glucose + oxidase	0
3. Dehydrogenase + glucose + cytochrome	7
4. Dehydrogenase + glucose + cytochrome + oxidase	101
5. Glucose + cytochrome + oxidase	18

It is clear that the only solution showing an appreciable oxygen uptake is that containing the complete system, glucose with its dehydrogenase, cytochrome and indophenol oxidase. (In some experiments, an oxidation of glucose has been induced by indophenol oxidase without added cytochrome. Keilin has found, however, that the heart-muscle indophenol oxidase always contains a small amount of cytochrome. The oxidation of glucose in presence of oxidase *plus* added cytochrome has always been much greater.)

In order to exclude the possibility that the results in this experiment might be due to glycolysis of glucose to lactic acid by the indophenol oxidase preparation, followed by oxidation of the lactic acid, the following experiment was carried out. The manometric experiment was performed exactly as before except that the undialysed dehydrogenase preparation was used and the temperature was 30°. At the same time, to each of four small conical flasks were added 3·5 cc. dehydrogenase and 1·65 cc. indophenol oxidase. 0·41 cc. 2*M* glucose + 0·62 cc. water were added to two flasks *A* and *A'*, while duplicate control flasks *B* and *B'* received 1·03 cc. water but no glucose. (The concentrations are the same as those used in the manometric experiments.) The flasks were quickly filled with nitrogen, stoppered and incubated for 3½ hours at 30°. 14 cc. of water and 6 cc. 20 % trichloroacetic acid were then added to each flask, and after removal of glucose by the copper-lime method, lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927]. The results were as follows:

	Lactic acid mg.
A with glucose	0·47
A' with glucose	0·47
B without glucose	0·42
B' without glucose	0·47

It is evident that no measurable quantity of lactic acid has been formed from the glucose. The manometric experiment which was carried out simultaneously at 30°, gave similar results to that described above, namely no appreciable oxygen uptake except where the complete system, glucose with its dehydrogenase, cytochrome and indophenol oxidase, was present. This shows

therefore that the oxygen uptake is due to the direct oxidation of glucose rather than to the oxidation of lactic acid formed by glycolysis of the glucose.

Discussion.

This appears to be the first time that a soluble glucose dehydrogenase has been isolated from the cell. The only reference in the literature to the extraction of an enzyme which oxidises glucose appears to be that of Müller [1929] who obtained a glucose oxidase from *Aspergillus niger*. This enzyme is entirely different in its properties from the enzyme which I have described. Müller's enzyme does not bring about the oxidation of glucose in presence of methylene blue and does in presence of oxygen. Like tyrosinase, therefore, it is an oxidase and not a dehydrogenase. The resistance of glucose dehydrogenase to toluene suggests that it is different from the glucose-oxidising enzyme which is present in suspensions of certain bacteria; Quastel and Wooldridge [1927] have found that in "resting" *B. coli* the enzyme which brings about the oxidation of glucose is completely inactivated by treatment with toluene.

I have not been able to find any example in the literature of an oxidising enzyme which can be filtered through kieselguhr and a fine porcelain candle without loss of activity. The lactic dehydrogenase prepared by Stephenson [1928] from *B. coli* could be filtered through kieselguhr but was completely inactivated by passing the solution through a porcelain candle. The observation that glucose dehydrogenase may be filtered through a fine porcelain candle is of interest as indicating that the activity of the enzyme is associated with molecules in true colloidal solution. The molecules constituting the enzyme or to which the enzyme is attached are doubtless large and extremely complex, but the enzyme activity is evidently not to be ascribed to a suspension of microscopic remnants of the broken cell. It has been shown that glucose dehydrogenase with glucose does not take up oxygen to any appreciable extent. Also, the indophenol oxidase preparation from washed heart muscle does not contain any glucose dehydrogenase. These two facts rendered the glucose dehydrogenase a particularly suitable enzyme for testing the ability of cytochrome *plus* indophenol oxidase to induce the aerobic oxidation of a substrate activated by an anaerobic dehydrogenase. The experiments described above, showing that in presence of glucose dehydrogenase, cytochrome and indophenol oxidase, glucose is oxidised by oxygen, afford the first direct demonstration of the co-operation of the oxidase and cytochrome with a dehydrogenase in bringing about the aerobic oxidation of the substrate of the last of these.

It is premature to speculate on the function in the body of glucose dehydrogenase until more is known about the distribution of the enzyme and until the oxidation product of glucose has been identified. The acid formed by the oxidation of glucose is now being investigated.

SUMMARY.

1. An enzyme (glucose dehydrogenase) has been obtained by saturating the water extract of acetone-liver with ammonium sulphate. The enzyme has been extracted from the liver of the ox, sheep, cat and dog.

2. The enzyme is obtainable in a perfectly clear, cell-free solution which can be filtered through a porcelain candle or through kieselguhr.

3. The enzyme brings about the oxidation of glucose in presence of methylene blue as hydrogen acceptor.

4. This anaerobic oxidation of glucose is not inhibited by KCN, NaF, toluene or iodoacetic acid.

5. Glucose is not oxidised in the presence of oxygen, an oxygen activator apparently being necessary.

6. The system glucose and glucose dehydrogenase has been combined with Keilin's cytochrome-indophenol oxidase system in presence of which the aerobic oxidation of glucose readily occurs, cytochrome acting as a carrier between active oxygen from the oxidase and active hydrogen from glucose *plus* its dehydrogenase.

7. The oxidation product of glucose has not yet been determined, but it appears to be an acid. (See Note below.)

The author is extremely grateful to Prof. Edward Mellanby for his kind interest in this work.

Part of the expense was defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

Bernheim (1928). *Biochem. J.* **22**, 1178.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Harrison (1931). *Biochem. J.* **25**, 1011.
Keilin (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
—— (1930). *Proc. Roy. Soc. Lond.* B **106**, 418.
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
Müller (1929). *Biochem. Z.* **213**, 211.
Quastel and Wooldridge (1927). *Biochem. J.* **21**, 159.
—— —— (1928). *Biochem. J.* **22**, 689.
Stephenson (1928). *Biochem. J.* **22**, 605.

Note added July 24th, 1931.

It has now been found that the oxidation product of glucose is gluconic acid. This has been isolated as the characteristic calcium salt, from which the pure phenylhydrazide has been prepared. The latter melted at 199°, the melting-point being unchanged by mixing with an authentic specimen. The cinchonine salt of the acid melted at 186° after recrystallisation from 95% alcohol. Gluconic acid appears to be the main, if not the sole, product of the oxidation. It is hoped shortly to publish these results in detail.

CXIV. SYNTHESIS OF THE *d*- AND *l*-3:4-DIHYDROXYPHENYLALANINES.

By CHARLES ROBERT HARINGTON and SYDNEY STEWART RANDALL.

From the Department of Pathological Chemistry, University College Hospital Medical School, London.

(*Received June 6th, 1931.*)

Although *dl*-3 : 4-dihydroxyphenylalanine has been synthesised on several occasions [Funk, 1911; Stephen and Weizmann, 1914; Harington and McCartney, 1927], the optically active forms of this amino-acid have not so far been artificially obtained. *l*-3 : 4-Dihydroxyphenylalanine was first isolated by Guggenheim [1913] from *Vicia faba*; renewed biological interest attaches to this amino-acid as the result of the recent work of Raper [1926], who has shown that it represents the first stage in the formation of melanin from tyrosine by the enzyme tyrosinase. It therefore appeared to us desirable to complete the synthesis of *l*-3 : 4-dihydroxyphenylalanine by resolution of the *dl*-amino-acid into its optically active components.

We attempted first to formylate the *dl*-amino-acid with the intention of subsequently resolving the formyl derivative by means of suitable alkaloidal salts. The formyl derivative turned out, however, to be an excessively soluble substance which was also difficult to handle owing to the extreme ease with which it was oxidised.

We then turned our attention to the preparation of a triacetyl derivative of dihydroxyphenylalanine which would be free from the objection of ready oxidisability. Having failed, in this case, to obtain any good result by the elegant method of acetylation with ketene, recently described by Bergmann [1930], we proceeded as follows. Protocatechuic aldehyde was condensed with acetylglycine in presence of acetic anhydride and sodium acetate. The azlactone formed in this reaction is much less stable than is generally the case, and on standing in contact with water is converted into β-3 : 4-diacetoxyphenyl-α-acetaminoacrylic acid. Reduction of this compound to the corresponding propionic acid derivative by means of sodium amalgam in the ordinary way was excluded by the ease with which the acetoxy-groups were hydrolysed in alkaline solution. It could, however, be effected readily and in good yield by a catalytic method. The resulting β-3 : 4-diacetoxyphenyl-α-acetaminopropionic acid was treated with one equivalent of hydrated brucine in concentrated alcoholic solution. Crystallisation set in very slowly and was not complete until a fortnight had elapsed. During the crystallisation a smell of ethyl acetate

developed and it was found in fact that the salt which had separated was the brucine salt of *l*-*β*: 3: 4-dihydroxyphenyl-*α*-acetaminopropionic acid, elimination of the O-acetyl groups having occurred during the protracted crystallisation. The salt of the corresponding *d*-acid was obtained from the mother-liquor.

The free acids, as was to be expected, were excessively soluble compounds and very readily oxidisable. They could not be isolated satisfactorily and the solutions recovered from the salts after removal of the alkaloid in the usual way were, therefore, submitted directly to hydrolysis with hydrochloric acid. In this way there were obtained preparations of the *d*- and *l*-amino-acids having respectively $[\alpha]_D + 11{\cdot}9°$ and $- 12{\cdot}0°$. The *l*-amino-acid corresponded in all its properties with a sample of the natural acid isolated from *Vicia faba*.

Experimental.

β-3 : 4-Diacetoxyphenyl-α-acetaminoacrylic acid. Protocatechuic aldehyde (21·6 g.), acetylglycine (18·4 g.) and freshly fused sodium acetate (12·8 g.) were ground together to form an intimate mixture. This was treated with freshly distilled acetic anhydride (60 cc.) and the whole was heated on the steam-bath for 2 hours. After cooling, the dark-coloured viscous solution was poured into cold water (750 cc.). The sticky yellow gum which separated hardened on keeping overnight. It was collected, washed with water and crystallised from ethyl acetate. A further crop was obtained by concentrating the ethyl acetate mother-liquor, stirring up the residue with water, keeping overnight and then proceeding as before. The total yield was 18 g. For analysis the acid was recrystallised from water, decoloration being effected with charcoal. It separated in glistening white plates. The air-dried material sintered at 115°–120° and melted at 183·4°. On heating at 100° it lost $1H_2O$ and the anhydrous product melted at 187–8°.

(Found for the anhydrous product: C, 56·1; H, 4·8; N, 4·4 %. $C_{15}H_{15}O_7N$ requires: C, 56·1; H, 4·7; N, 4·4 %. 300 mg. of the air-dried material lost 17·3 mg. in 3 hours at 100°; whence loss of weight = 5·8 %; calculated for $1H_2O$ 5·3 %.)

β-3 : 4-Diacetoxyphenyl-α-acetaminopropionic acid. The above-described unsaturated acid (28 g.) was dissolved in warm water (4000 cc.) The solution was cooled to the ordinary temperature, and, after addition of 1 g. of palladium chloride (dissolved in water with the aid of the minimum amount of hydrochloric acid), was shaken in an atmosphere of hydrogen. The uptake of hydrogen ceased after 2 hours, by which time 2600 cc. had been absorbed. On keeping the solution overnight the catalyst flocculated and could easily be removed by filtration. The filtrate was digested with a suspension of well-washed silver carbonate; after removal of the precipitate the solution was freed from excess of silver with hydrogen sulphide and concentrated under diminished pressure to about 800 cc. when separation of crystalline material commenced. The crystals were collected after the solution had stood overnight and amounted to 17·4 g.; a further crop of 3 g. was obtained by concentrating the mother-liquor to about 75 cc.

The acid was recrystallised from water from which it separated in small colourless blunt prisms, M.P. 171–172°. It is readily soluble in hot water, sparingly so in cold. It does not absorb bromine nor does it decolorise alkaline permanganate immediately.

(Found: C, 55·6; H, 5·3; N, 4·4 %. $C_{15}H_{17}O_7N$ requires: C, 55·7; H, 5·3; N, 4·3 %.)

Brucine l-β-3 : 4-dihydroxyphenyl-α-acetaminopropionate. dl-β-3 : 4-Diacetoxyphenyl-α-acetaminopropionic acid (27 g.) and brucine (38·5 g. of the hydrated alkaloid) were dissolved together in absolute alcohol (270 cc.) and the solution was set aside. Crystallisation commenced in about 24 hours. After 14 days the solid, which appeared not to be increasing in amount any further, was filtered off, washed with a little alcohol and dried. The yield was 19 g. The salt was recrystallised from 95 % alcohol from which it separated in colourless needles containing $2H_2O$; attempts to remove the water of crystallisation failed owing to decomposition of the salt at the high temperature required; on heating in a capillary tube it sintered at 157°, and melted with effervescence at 176°.

It was readily soluble in water, sparingly so in absolute alcohol, and insoluble in other organic solvents.

After two recrystallisations the rotation became constant

$$\alpha^{20°}_{5461} = -1{\cdot}89° \ (c = 2{\cdot}04, \ l = 2);$$

whence

$$[\alpha]^{20°}_{5461} = -46{\cdot}3°.$$

The strong smell of ethyl acetate which had developed during the slow separation of this salt had suggested the possibility of de-acetylation. That such de-acetylation had indeed occurred was shown by the analysis of the salt, the figures agreeing well with those required by the brucine salt of *N*-acetyl-3 : 4-dihydroxyphenylalanine with $2H_2O$. (Found: C, 61·48; H, 6·47; N, 6·64 %. $C_{34}H_{39}O_9N_3$, $2H_2O$ requires: C, 61·1; H, 6·43; N, 6·28 %.) Further, the salt gave with ferric chloride in neutral aqueous solution an intense green coloration which passed into reddish-purple on addition of sodium bicarbonate, indicating that the adjacent hydroxyl groups attached to the benzene ring were free.

Brucine d-β-3 : 4-dihydroxyphenyl-α-acetaminopropionate. The alcoholic mother-liquor after removal of the crude insoluble brucine salt was concentrated under diminished pressure to a syrup. This was kept for a week, during which time it partly solidified. The pasty mass was boiled out three times in succession with ethyl acetate and then triturated with ether until it became wholly granular. The crude dried material (24 g.) was crystallised from water, in which it was very soluble at the boiling point but sparingly so in the cold. After two recrystallisations the optical rotation became constant

$$\alpha^{20°}_{5461} = +0{\cdot}31° \ (c = 2{\cdot}04, \ l = 2);$$

whence

$$[\alpha]^{20°}_{5461} = +7{\cdot}6°.$$

The salt formed long narrow prisms containing $2H_2O$, which sintered at 150° and melted with effervescence at 154°.

(Found: C, 61·46; H, 6·51; N, 6·35 %. $C_{34}H_{39}O_9N_3$, $2H_2O$ requires: C, 61·1; H, 6·43; N, 6·28 %.)

d-3 : 4-Dihydroxyphenylalanine. The laevorotatory brucine salt described above was dissolved in water and the solution, cooled in ice, was treated with excess of saturated aqueous barium hydroxide. The mixture was stirred vigorously with a glass rod for about 5 minutes whilst still in the ice in order to accelerate the separation of the alkaloid. This was filtered off rapidly and the last traces were removed by extraction with chloroform, the air in the separating-funnel being displaced with hydrogen. With as little delay as possible the aqueous solution was treated with just sufficient dilute sulphuric acid to precipitate all the barium, brought to the boil and filtered. The filtrate was concentrated under diminished pressure to about 15 cc. for every 5 g. of salt taken, treated with strong hydrochloric acid to make a final concentration of 10 % HCl and boiled under reflux for 1 hour. The solution was then evaporated to dryness under diminished pressure; the residue was dissolved in a little water, and ammonia was cautiously added, under a layer of light petroleum, until present in slight excess. The whole was then rapidly evaporated to dryness under diminished pressure in a stream of hydrogen. The crystalline residue was purified by dissolving in boiling water containing a trace of sulphur dioxide and treatment with charcoal; the filtered solution deposited stout colourless prisms of the amino-acid on cooling. The amino-acid melted at 282° with decomposition. In 5·01 % solution in *N* hydrochloric acid the observed rotations were $\alpha_{5461} + 1{\cdot}33°$ and $\alpha_D + 1{\cdot}20°$ respectively in a 2 dm. tube; whence $[\alpha]_{5461} = + 13{\cdot}2°$ and $[\alpha]_D = + 11{\cdot}9°$.

l-3 : 4-Dihydroxyphenylalanine. This was obtained in a precisely similar manner from the alcohol-soluble dextrorotatory brucine salt and formed colourless prisms, M.P. 282° (decomp.). In 5·04 % solution in *N* hydrochloric acid the observed rotations in a 2 dm. tube were $\alpha_{5461} - 1{\cdot}30°$ and $\alpha_D - 1{\cdot}21°$ respectively, whence $[\alpha]_{5461} = - 12{\cdot}9°$ and $[\alpha]_D = - 12{\cdot}0°$. A sample of natural *l*-3 : 4-dihydroxyphenylalanine, kindly supplied by Dr M. Guggenheim, had M.P. 281° (decomp.); this M.P. was unaltered by admixture with the synthetic material. The natural acid had $[\alpha]_{5461} - 13{\cdot}6°$ and $[\alpha]_D - 12{\cdot}0°$ ($c = 5{\cdot}3$, and $l = 2$). The identity of the two acids is, therefore, established.

REFERENCES.

Bergmann (1930). *Ber. deutsch. chem. Ges.* **63**, 437.
Funk (1911). *J. Chem. Soc.* **99**, 554.
Guggenheim (1913). *Z. physiol. Chem.* **88**, 276.
Harington and McCartney (1927). *Biochem. J.* **21**, 852.
Raper (1926). *Biochem. J.* **20**, 735.
Stephen and Weizmann (1914). *J. Chem. Soc.* **105**, 1152.

CXV. THE ISOLATION OF *d*-3:5-DIIODOTYROSINE FROM THE THYROID GLAND BY THE ACTION OF PROTEOLYTIC ENZYMES.

By CHARLES ROBERT HARINGTON and SYDNEY STEWART RANDALL.

From the Department of Pathological Chemistry, University College Hospital Medical School, London.

(*Received June 30th, 1931.*)

About two years ago the present authors [1929] described the isolation of *dl*-3 : 5-diiodotyrosine by means of graduated hydrolysis of the thyroid gland with barium hydroxide. The isolation was repeated shortly afterwards by Foster [1929] who used a simpler method and obtained a better yield. Although in the previous work every precaution was taken to exclude the possibility of artificial production of diiodotyrosine during the course of the isolation, and although such artificial production was in any case very unlikely to occur, it remained nevertheless desirable to complete the demonstration of the natural occurrence of diiodotyrosine in the thyroid by its isolation by gentler means and in the optically active condition.

Promising material for this investigation offered itself in the various mother-liquors containing acid-soluble organically combined iodine which were obtained as by-products in the course of the work of Harington and Salter [1930] on the isolation of *l*-thyroxine by the enzymic digestion of thyroid gland.

As a first step the experience gained in previous work on diiodotyrosine was utilised to effect a preliminary concentration of the iodine-containing compounds present in the above-mentioned mother-liquors. Thus the mother-liquor obtained after removal of the material insoluble at p_H 5·0, following on the peptic digestion of crude iodothyreoglobulin, contained much iodine, a considerable proportion being present as iodide but a definite amount being still in organic combination. After preliminary clearing of this solution with lead acetate, practically the whole of the iodine was precipitated with silver nitrate in neutral or faintly alkaline solution. The organic iodine compounds were separated from iodide by extraction of the precipitated silver salts with dilute nitric acid and re-precipitated by addition of ammonia; decomposition of the re-precipitated silver salts in the usual manner yielded a solution of the organic iodine compounds in high concentration which was subjected to further digestion with trypsin.

After the tryptic digestion was complete the solution was subjected to a

process of fractionation closely similar to that employed in the previous work [Harington and Randall, 1929] on the isolation of diiodotyrosine, yielding finally a soluble gummy material containing much iodine and giving an intense reaction for diiodotyrosine with nitrous acid and ammonia, but at the same time still giving a biuret reaction.

A product with similar characteristics was obtained by analogous concentration of the organic iodine-containing compounds which remained in solution after removal of the thyroxine-containing fraction of the tryptic digestion products of iodothyreoglobulin [*cf.* Harington and Salter, 1930].

The final successful isolation of diiodotyrosine was achieved by subjecting the combined solution of a number of such end-products of tryptic digestion to the action of an active preparation of erepsin. After this treatment it was possible by a simple fractionation to separate diiodotyrosine in the pure condition.

The losses involved at the various stages of the procedure outlined above were very heavy and the amount of diiodotyrosine finally obtained has no quantitative significance. Evidence was offered previously [Harington and Randall, 1929] bearing on the probable amount of diiodotyrosine actually present in the thyroid, and the suggestions made at that time have since been largely confirmed by the improved yield obtained by Foster [1929]. The present work is to be regarded as the final evidence that *d*-diiodotyrosine does actually occur as a constituent amino-acid of the characteristic protein of the thyroid.

Experimental.

Isolation of d-*3 : 5-diiodotyrosine from the acid-soluble fraction of peptic digestion products from iodothyreoglobulin.*

The filtrate from a peptic digestion of crude iodothyreoglobulin [*cf.* Harington and Salter, 1930], amounting to 7200 cc. and containing in all 1·986 g. of iodine, was treated with a concentrated aqueous solution of 350 g. of lead acetate and then with sodium hydroxide until just neutral to litmus. The solution was filtered, and the filtrate, containing 1·4 g. of total iodine, was made faintly alkaline to litmus with ammonia and treated with silver nitrate to complete precipitation. The insoluble silver salts were filtered off, suspended in about 800 cc. of water, and treated with freshly boiled out nitric acid until the suspension was permanently acid to Congo red. The silver halides were removed as rapidly as possible by filtration, and the organic silver salts were re-precipitated from the filtrate by the cautious addition of ammonia until present in slight excess. The precipitate was collected, thoroughly washed with water, suspended in 1000 cc. of water and decomposed with hydrogen sulphide. The filtrate from the silver sulphide, containing 423 mg. of iodine was concentrated under diminished pressure to 360 cc.

This solution, which was slightly acid, was brought to p_H 8·0 by addition of sodium hydroxide and was treated with 0·8 g. of trypsin (Merck) dissolved in

10 cc. of water. Toluene was added as a preservative and the solution was placed in an incubator at 38°. At the commencement of the digestion the total N amounted to 20·0 mg./cc. and the amino-N to 1·0 mg./cc. After 48 hours the amino-N had increased to 2·65 mg./cc.; a further addition of 0·8 g. of trypsin was made at this point, which, in the course of another 24 hours, increased the amino-N to 3·01 mg./cc. At this stage the digestion was interrupted; the solution was diluted to 2 litres, neutralised and brought to the boil. A saturated solution of uranium acetate was then added until no further precipitation occurred. The precipitate was removed and the excess of uranium was thrown out with ammonia. The filtered solution was concentrated to 200 cc. and contained 271 mg. of iodine. Silver nitrate was added to complete precipitation, and the insoluble silver salts were fractionated by extraction with nitric acid as described above.

The solution obtained by recovery from the organic silver salts contained 174 mg. of iodine. It was concentrated to 200 cc. and extracted by shaking out 9 times at 70° with purified (aldehyde-free) butyl alcohol. The butyl alcohol extracts were evaporated to dryness under diminished pressure, the evaporation being several times repeated after addition of water. The residue contained 128 mg. of iodine; it was dissolved in water (600 cc.) and the clearing process with uranium acetate was repeated. After removal of the uranium the solution, containing 109 mg. of iodine, was concentrated under diminished pressure to 140 cc. and precipitated with basic lead acetate. After standing overnight the precipitate was filtered off, suspended in water and decomposed with hydrogen sulphide. The filtrate from the lead sulphide, when concentrated, yielded a yellow gum which contained 67 mg. of iodine all in organic combination. The material gave a strong colour reaction for diiodotyrosine with nitrous acid and ammonia, but gave also a biuret reaction and could not be induced to crystallise.

The residues from two such experiments, containing in all 142 mg. of iodine, were therefore united in solution in 30 cc. of water; 60 cc. of an active erepsin extract were added and the mixture was brought to p_H 8·0, saturated with toluene and placed in an incubator at 38°. At the commencement of the digestion the mixture contained total N 2·18 mg./cc. and amino-N 0·51 mg./cc.; the digestion was interrupted after 67 hours when the amino-N had reached 0·96 mg./cc. and the rate of increase had become very low.

The solution was diluted with 50 cc. of water and treated with excess of silver nitrate. The insoluble silver salts were filtered off, washed and decomposed with hydrogen sulphide. The filtrate from the silver sulphide (containing 124 mg. of iodine) was freed from hydrogen sulphide and cleared with uranium acetate; after removal of excess of uranium the solution was concentrated to 80 cc. and contained 100 mg. of iodine. Basic lead acetate was added in slight excess, and after standing overnight the precipitate was collected, washed, suspended in water and decomposed with hydrogen sulphide. The lead sulphide was thoroughly boiled out several times with water to extract adsorbed diiodotyrosine. The filtrate and extracts from the lead sulphide, which con-

tained 88 mg. of iodine, were concentrated to a low bulk under diminished pressure.

On keeping overnight an amorphous precipitate, admixed with nodules of colourless needles, had separated. The latter were collected and the mother-liquor was cleared on the centrifuge and concentrated further, when a second crop of crystals was obtained. A still further amount of crystals was obtained by dissolving the amorphous precipitate in boiling 50 % acetic acid and allowing the solution to cool. The whole of the crystalline material was collected and recrystallised from 50 % acetic acid, yielding 77 mg. of prismatic needles. (Found: N, 3·18 %; calc.: N, 3·2 %.) The compound gave the characteristic colour reaction with nitrous acid and ammonia with intensity; it had M.P. 194° (decomp., uncorr.) and this M.P. was unchanged by admixture with a sample of *d*-3 : 5-diiodotyrosine prepared by the iodination of *l*-tyrosine. In 3·64 % solution in *N* hydrochloric acid, using a 0·5 dm. tube, the observed rotations of the material from the thyroid were $\alpha_{5461} + 0{\cdot}07°$ and $\alpha_D + 0{\cdot}05°$; whence $[\alpha]_{5461} + 3{\cdot}85°$ and $[\alpha]_D + 2{\cdot}75°$. Abderhalden and Guggenheim [1908] record $[\alpha]_D + 3{\cdot}0°$ for the acid dissolved in 4 % hydrochloric acid.

Isolation of d-*3 : 5-diiodotyrosine from the acid-soluble fraction of tryptic digestion products of iodothyreoglobulin.*

The filtrates obtained by removal of all material insoluble at p_H 5·0 after the tryptic digestion of iodothyreoglobulin [*cf.* Harington and Salter, 1930] were subjected to a process of fractionation similar to that just described for the tryptic digest of the acid-soluble peptic products. The procedure in one such instance is summarised below.

	Iodine (mg.)
Tryptic filtrate (3600 cc.)	481
After clearing with uranium acetate	348
After precipitating with silver nitrate, fractionation of silver salts and recovery	168
After butyl alcohol extraction	153
After second uranium treatment	119

The gummy residues from four such experiments, containing in all 318 mg. of iodine, were united; one-half was subjected to digestion with erepsin at p_H 8·0 for 93 hours. The digest was worked up as before and yielded finally 150 mg. of crude crystalline *d*-3 : 5-diiodotyrosine.

Note on preparation of active erepsin extract.

The following method has been found convenient for the preparation of active erepsin extract.

The mucous membrane of the small intestine of a recently killed dog was scraped off with a spatula and ground up with sand in a mortar under 5 times its weight of 66 % glycerol. After standing overnight the mixture was diluted with 4 vols. of water and rapidly centrifuged. The extract was submitted to dialysis under diminished pressure in an apparatus of the type described by Sørensen [1918] until free from glycerol and was finally concentrated by

filtration through the collodion bag until the volume was one-half that of the original extract. A slight precipitate was removed by filtration through ordinary filter-paper. The resulting solution was kept at 0° in presence of toluene, but was used as soon as possible. It contained a very active erepsin, being capable of hydrolysing glycylglycine quantitatively in a few hours.

REFERENCES.

Abderhalden and Guggenheim (1908). *Ber. deutsch. chem. Ges.* **41**, 1237.
Foster (1929). *J. Biol. Chem.* **83**, 345.
Harington and Randall (1929). *Biochem. J.* **23**, 373.
—— and Salter (1930). *Biochem. J.* **24**, 456.
Sørensen (1918). *Z. physiol. Chem.* **103**, 1.

CXVI. THE METABOLISM OF IODOBENZENE AND *p*-IODOPHENYLMERCAPTURIC ACID IN THE RAT AND THE RABBIT.

By NORMAN RISBOROUGH LAWRIE.

From the Biochemical Laboratory, Cambridge.

(*Received June 12th, 1931.*)

The present work was undertaken as a preliminary step in the investigation of the experimental haemoglobinuria described by Callow and Hele [1927]. These workers found that *p*-bromo- and *p*-chloro-phenylmercapturic acids, whether given by the mouth or subcutaneously, cause a transient haemoglobinuria in the dog. An attempt was made to find a small animal which would show a similar response to dosage with mercapturic acids. This attempt has not so far been successful; but in the course of the work certain interesting features of the sulphur metabolism of rats and rabbits were displayed. The possibility of mercapturic acid formation from iodobenzene and the effect of dosage with *p*-iodophenylmercapturic acid were studied in both these animals.

The ability of the rabbit to synthesise *p*-bromophenylmercapturic acid has been shown by Abderhalden and Wertheimer [1925], and some evidence in favour of the occurrence of this synthesis in the rabbit has been brought forward by Rhode [1922], and by Shiple, Muldoon and Sherwin [1924] in the case of the pig. The evidence suggesting a synthesis of mercapturic acid by the rabbit, brought forward by Rhode, was very inconclusive, being founded solely upon the diminished synthesis of ethereal sulphate from monohalogen benzene derivatives when cystine was given simultaneously.

Evidence in favour of the oxidation of mercapturic acid to ethereal sulphate has been adduced by Shiple, Muldoon and Sherwin [1924] for the case of the pig, and by Rose, Shiple and Sherwin [1924] for the case of the rabbit. On the other hand, previous work in this laboratory by Coombs and Hele [1927] indicated that this oxidation does not occur in the pig, and a criticism of the conclusions drawn by Sherwin and his co-workers is given in their paper.

No case of haemoglobinuria following the administration of a mercapturic acid has been described, other than the observations of Callow and Hele quoted above; nor has haemoglobinuria been obtained, either in the rat or the rabbit, in the course of the present work.

Experimental.

I. *Experiments upon the rat.*

An inbred, piebald strain was used. The degree of toxicity of iodobenzene and *p*-iodophenylmercapturic acid for the rat is indicated by the

following data. It was found that iodobenzene could be given to adult male rats, by subcutaneous injection, in doses of 1·5 mg. per g. of body weight, every three days, for 12–18 days, before any marked deterioration in the animal's general condition occurred. A single dose of this size was lethal within 48 hours when given intraperitoneally. *p*-Iodophenylmercapturic acid was given as the aqueous solution of its sodium salt, and a dose of 1 mg. per g. of body weight was lethal in over 50 % of cases. A dose of 0·6 mg. per g. of body weight was well tolerated when given subcutaneously. Mr Innes, of the Department of Animal Pathology, Cambridge, has kindly submitted the kidneys, livers and intestines of animals used in the above experiments to histological examination, and found no gross pathological changes.

In all experiments upon the rat, the following synthetic diet, made into a paste with distilled water, was fed *ad lib.*

"Light white casein"	23 %
Starch	40 %
Cane sugar	17 %
Salt mixture	5 %
Palm kernel oil	15 %

Cod-liver oil and marmite were added to the above diet in the proportions of 2·5 % and 7·5 % respectively. In each experiment two Hopkins metabolism cages were set up, each cage containing two adult male rats. Each morning, the urine which had been passed during the preceding 24 hours, from all four rats, was mixed with the rinsings from the collecting apparatus, made up to a suitable volume with distilled water and filtered before analysis.

Analytical Methods.

Total sulphur, total sulphate and inorganic sulphate were determined by the methods used in this laboratory for the analysis of dog's urine [Hele, 1924]. The ethereal sulphates and neutral sulphur were obtained by difference. Iodine was estimated by the method of Kendall [1920] with the exception that one incineration only was required in order to destroy completely the organic matter present in 10 cc. of urine. Iodine estimated in alkaline solutions of *p*-iodophenylmercapturic acid was found in approximately the proportion of 92 % of that present.

Exp. 1. In this experiment iodobenzene was twice given subcutaneously. The results of the sulphur analyses are recorded graphically in Fig. 1. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I A. Owing to the small quantity of urine available for analysis no satisfactory estimations of neutral sulphur and ethereal sulphate were obtained for the 4th day of this experiment.

Exp. 2. *p*-Iodophenylmercapturic acid was given once mixed with the food, when about one-third of its iodine was detected in the urine; and once by stomach tube. The results of the sulphur analyses are recorded graphically in

Fig. 2. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I B.

Table I.

Experiment and dose	Period in days	Neutral S daily av. mg. S	Ethereal SO_4 daily av. mg. S	Total extra neutral S mg. S	Total extra ethereal SO_4 mg. S	% dose excreted as sulphur compound	% dose excreted as iodine compound
A. 4 ♂ Rats. Figures calculated per rat							
Average figures for 9 normal days	1–6, 10–13	6·8	0·9	—	—	—	—
On 7th day 0·18 g. C_6H_5I subcutaneously	7–9	—	—	12·4	3·5	56	102
On 14th day 0·34 g. C_6H_5I subcutaneously	14–19	—	—	38·9	4·0	80	102
B. 4 ♂ Rats. Figures calculated per rat							
Average figures for 12 normal days	1–8, 13–16	7·2	1·0	—	—	—	—
On 9th day 0·75 g. *p*-iodophenylmercapturic acid mixed with food	9–12	—	—	26·5	nil	43	33
On 17th day 0·20 g. ditto by stomach tube	17–18	—	—	12·5	nil	71	32
C. Rabbit I. ♀							
Average figures for 28 normal days	1–10, 13–18, 25–32, 35–38	180*	13	—	—	—	—
On 11th day 5·4 g. C_6H_5I by stomach tube	11–12	—	—	nil*	280	33	54
On 19th day ditto subcutaneously	19–24	—	—	92*	182	32*	98
On 33rd day 3·0 g. and on 34th day 2·0 g. *p*-iodophenylmercapturic acid by stomach tube	33–34	—	—	234*	nil	53*	64
D. Rabbit II. ♂							
Average figures for 12 normal days	1–12	8·2	3·0	—	—	—	—
On 13th and 14th days 2 g. *p*-iodophenylmercapturic acid by stomach tube	13–14	—	—	312	nil	89	73

* The significance of these figures is uncertain, owing to the irregularity of the neutral sulphur excretion.

Exp. 3. Isolation of p-*iodophenylmercapturic acid from rat urine.* Sixteen adult male rats, caged in Hopkins metabolism cages, were used in this

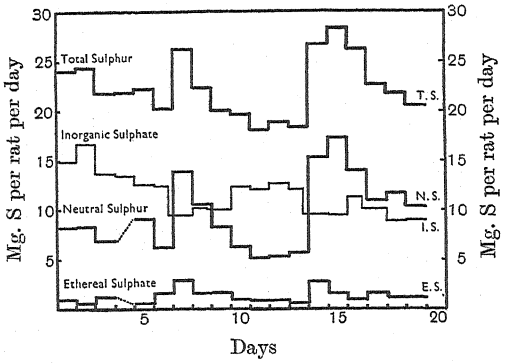


Fig. 1. Dosage. On 7th day, 0·18 g. iodobenzene per rat, subcutaneously.
„ 14th „ 0·34 „ „ „

experiment. Urine was collected daily, and stored over chloroform. The full synthetic diet described above was fed throughout. Each rat received 0·36 g. of iodobenzene subcutaneously every three days, until a total of 34·6 g. had been given. Five litres of urine were obtained, and worked up in two batches of $2\frac{1}{2}$ litres each.

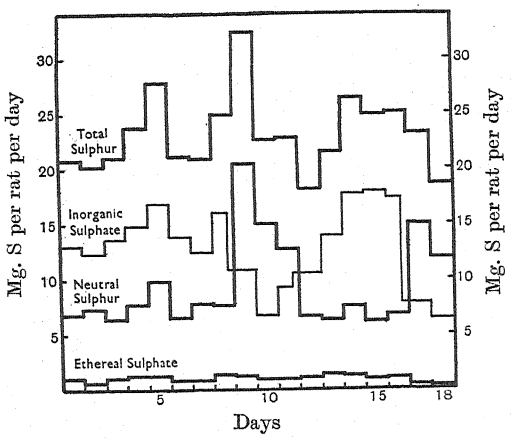


Fig. 2. Dosage. On 9th day, 0·75 g. *p*-iodophenylmercapturic acid per rat, mixed with food.
„ 17th „ 0·20 „ „ „ given by stomach tube.

Treatment of first batch. This batch was separated into excess chloroform, precipitate, and filtrate. Analysis showed that nearly all the iodine was in the filtrate. An attempt was made to isolate *p*-iodophenylmercapturic acid from this fraction by Baumann's method, which had given good results with dog's urine in this laboratory. This method, however, failed to remove coloured impurities, and only a very small yield of pigmented material melting at 151° and decomposing at 187° was obtained. Attempts to decolorise this material

showed that the mercapturic acid was strongly adsorbed on to Merck's medicinal charcoal, and this property was utilised in the treatment of the second batch.

Treatment of second batch. The urine was filtered, and 600 cc. of the filtrate used in preliminary trials of the method. The remainder was acidified with hydrochloric acid and allowed to stand for eight days. The precipitate obtained was dissolved in dilute ammonia, and the solution shaken with Merck's medicinal charcoal until the filtrate gave no precipitate on acidification. The charcoal was then dried at 37° and extracted with successive 100 cc. portions of 97 % alcohol, until on evaporation of the alcohol only traces of solid residue were obtained. Approximately 20 g. of charcoal and 2 litres of alcohol were required.

The preliminary precipitation with hydrochloric acid and solution of this precipitate in ammonia removed 1·6 g. of the total iodine present in batch 2 of urine. Of this, 1·39 g. was absorbed on the charcoal. The extraction with alcohol removed 1·06 g. from the charcoal, and the final purified product contained 0·61 g. of iodine, thus giving a yield of mercapturic acid of 8·2 % from the iodobenzene injected.

Analysis of this product gave the following figures:

	N	S	I
Found	3·84	9·06	35·4
Theoretical for *p*-iodophenylmercapturic acid	3·84	8·77	34·8

The melting-point (152°) was unchanged on mixing with various proportions of pure *p*-iodophenylmercapturic acid, prepared in this laboratory from the urine of dogs which had been fed with iodobenzene.

II. *Experiments upon the rabbit.*

The rabbit was found to tolerate iodobenzene very readily. A dose as high as 5 g. given by subcutaneous injection to a rabbit weighing 3 kg. produced only a transient diminution of appetite. A similar slight reaction was observed in the case of the doe rabbit used in Exp. 4, after giving, by stomach tube, a dose of *p*-iodophenylmercapturic acid of 1·9 mg. per g. body weight. This rabbit was fed upon a diet of oats and cabbage. The buck rabbit used in Exp. 5, and fed upon a diet of sugar-beet, died 24 hours after receiving, by stomach tube, the second instalment of a dose of *p*-iodophenylmercapturic acid of 2·1 mg. per g. body weight.

The diet used in Exp. 4 consisted of 150 g. minced cabbage and 20 g. Sussex oats, given morning and evening. In Exp. 5, however, this diet was changed, in order to decrease the output of urinary sulphur. In this experiment the rabbit received 50 g. of sliced sugar-beet morning and evening.

In both experiments, the rabbit was kept in a cage with a wire floor, set in a large zinc funnel. Beneath the outlet of this funnel was placed a Hopkins separator of rather larger size than that used in rat work. Efficient separation

of urine and faeces was thus obtained. On every alternate morning the collecting apparatus was rinsed with distilled water, and the combined rinsings and urine were made up to a suitable volume, acidified with acetic acid to dissolve the copious phosphatic precipitate usually present, and filtered before analysis. In the case of the buck used in Exp. 5, catheterisation was carried out at the end of each two-day period of collection of urine. This procedure was not practicable in the case of the doe used in Exp. 4.

The same analytical methods were used as in the experiments upon the rat. Nitrogen was estimated by the Kjeldahl method.

Exp. 4. An adult doe rabbit, weighing approximately 2·5 kg. was used in this experiment. Iodobenzene was given once by stomach tube and once by subcutaneous injection. *p*-Iodophenylmercapturic acid was given once by stomach tube. The results of the sulphur analyses are shown graphically in Fig. 3. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I C.

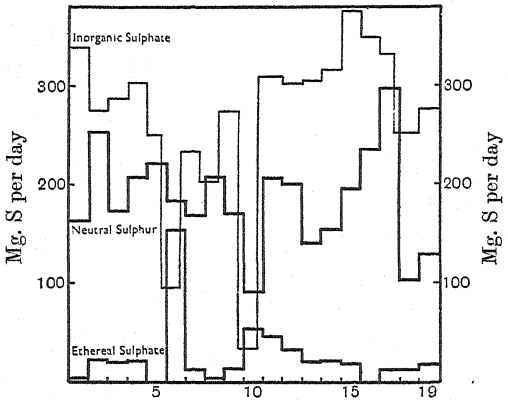


Fig. 3. Rabbit 1.

Dosage. On 1st day of 6th period, 5·4 g. iodobenzene by stomach tube.
" " " 10th " " " subcutaneously.
" " " 17th " 3·0 g. *p*-iodophenylmercapturic acid by stomach tube.
" 2nd " 17th " 2·0 " " " "

Exp. 5. An adult buck rabbit, weighing approximately 2·0 kg. was used in this experiment. *p*-Iodophenylmercapturic acid was given by stomach tube.

Particulars of this experiment are given in Table I D.

Exp. 6. In order to determine whether the rabbit was capable of synthesising a mercapturic acid from iodobenzene, to an extent too small to be detected by estimations of the neutral sulphur, the following experiment was carried out.

As a preliminary test, a rabbit was injected subcutaneously with 3·6 g. of iodobenzene. The urine passed during the next six days was treated as in the case of the second batch of rat urine in Exp. 3. About 50 mg. of somewhat impure *p*-iodophenylmercapturic acid were obtained, having M.P. 148°–149°, and giving the following analytical figures: N, 3·64; S, 9·06; I, 34·8.

Six adult doe rabbits were then placed upon a diet of carrot and oats, fed *ad lib.*, and housed in metabolism cages. Each rabbit received 3·6 g. iodobenzene subcutaneously and five days later a further injection of 3·6 g. The urine was collected daily for ten days after the first injection and preserved over chloroform. This urine was rendered distinctly alkaline to litmus with ammonia, and the phosphate precipitate filtered off. Its volume was then 5600 cc., and it contained about 10 g. of iodine. Treatment of this material according to the method described in Exp. 3 yielded 0·64 g. of impure *p*-iodophenylmercapturic acid, having M.P. 149°–150°. After purification by recrystallisation of the ammonium salt, the melting-point was 150°, and analysis gave the following figures: N, 3·70; S, 8·88; I, 34·75.

Discussion of Results.

I. *Experiments upon the rat.* Fig. 1 shows that the chief change brought about in the sulphur partition by a dose of iodobenzene is an increase in the neutral sulphur and total sulphur fractions. The ethereal sulphate shows only a slight increase. A large increase in the excretion of neutral sulphur, such as that occurring after the second dose of iodobenzene, may have been due, in part at least, to toxic effects of the dose. This possibility is shown more strikingly in the experiments with mercapturic acid (Fig. 2). Dosage with *p*-iodophenylmercapturic acid leads to an increase in the neutral sulphur excretion greater than that which would correspond to the mercapturic acid excreted, as judged by the iodine analyses. This excess of neutral sulphur appeared to be directly related to the severity of the signs of poisoning shown by the rats. In no case did dosage with *p*-iodophenylmercapturic acid cause haematuria or haemoglobinuria in the rat. The indication of mercapturic acid synthesis given by Exp. 1 was followed up in Exp. 3 by isolation of *p*-iodophenylmercapturic acid from rat urine. Thus the metabolism of iodobenzene in the rat is similar to its metabolism in the dog; but the rat differs from the dog in that it shows no haemoglobinuria after dosage with mercapturic acid.

II. *Experiments upon the rabbit.* Administration of iodobenzene to the rabbit, either orally or subcutaneously, causes a large increase in the ethereal sulphate output and a corresponding drop in the inorganic sulphate. No indication of mercapturic acid formation is given by the neutral sulphur figures (Fig. 3), although the conversion of a very small fraction of injected iodobenzene into *p*-iodophenylmercapturic acid is conclusively demonstrated in Exp. 6. The large fall in the excretion of all sulphur fractions, except ethereal sulphate, on the 10th day of Exp. 4 is accounted for by the fact that the animal refused its food for one day after this injection.

The effect of giving *p*-iodophenylmercapturic acid to rabbits is shown in Figs. 3 and Table I C and D. Both experiments show clearly that no appreciable oxidation of this substance to ethereal sulphate has occurred. This result contrasts with the findings of Sherwin and his co-workers, who stated that the rabbit oxidises *p*-bromophenylmercapturic acid to ethereal sulphate to the

extent of 23 % to 30 % [Rose, Shiple and Sherwin, 1924]; and that the pig is capable of oxidising phenyl-, *p*-chlorophenyl- and *p*-bromophenyl-mercapturic acids to ethereal sulphate to the extent of 38 %, 43 % and 23 % respectively. Previous work in this laboratory [Coombs and Hele, 1927], however, indicated that neither *p*-bromophenyl- nor *p*-iodophenyl-mercapturic acid could be oxidised to ethereal sulphate by the pig. The rise in the neutral sulphur excretion after giving *p*-iodophenylmercapturic acid is not well shown in Fig. 3 (Exp. 4) on account of the large and irregular quantities of neutral sulphur excreted throughout this experiment. In Exp. 5, a diet was chosen which gave a very low sulphur excretion, and the rise in neutral sulphur is very evident. In this experiment the rise in neutral sulphur is in excess of that which would correspond with the iodine excretion. This condition is doubtless due to the severe poisoning which occurred in the case of this animal. No haematuria or haemoglobinuria was observed in these experiments upon the rabbit.

Summary.

(1) It has been shown that the rat is able to synthesise *p*-iodophenylmercapturic acid from iodobenzene; is unable to oxidise that mercapturic acid to ethereal sulphate; and does not develop haemoglobinuria after the administration of that mercapturic acid.

(2) The rabbit is unable to oxidise *p*-iodophenylmercapturic acid to ethereal sulphate. It does not develop haemoglobinuria after the administration of that mercapturic acid. It is able to convert a very small fraction of injected iodobenzene into *p*-iodophenylmercapturic acid.

I wish to express my gratitude to Dr T. S. Hele for his interest and advice in this work. The expenses of this research were in part defrayed by a grant to Dr Hele from the Government Grant Committee of the Royal Society.

REFERENCES.

Abderhalden and Wertheimer (1925). *Pflüger's Arch.* **207**, 215; **209**, 611.
Callow and Hele (1927). *Biochem. J.* **21**, 606.
Coombs and Hele (1927). *Biochem. J.* **21**, 611.
Hele (1924). *Biochem. J.* **18**, 586.
Kendall (1920). *J. Biol. Chem.* **43**, 149.
Rhode (1922). *Z. physiol. Chem.* **124**, 15.
Rose, Shiple and Sherwin (1924). *Amer. J. Physiol.* **69**, 518.
Shiple, Muldoon and Sherwin (1924). *J. Biol. Chem.* **60**, 59.

CXVII. OBSERVATIONS ON SOME ALCOHOL-SOLUBLE PROTEINS FROM MILK PRODUCTS.

By LESLIE ALFRED ALLEN.

From the National Institute for Research in Dairying, The University, Reading.

(*Received June 19th, 1931.*)

The presence of alcohol-soluble proteins in milk or cheese has been reported by several workers. Weidemann [1882] isolated from Emmenthal cheese by extraction with boiling ethyl alcohol a substance which he called caseoglutin. This was confirmed by Rose and Schulze [1884] and Benecke and Schulze [1887]. Winterstein and Thöni [1902], Winterstein [1904] and Bisegger [1907], also working with Emmenthal cheese, concluded that caseoglutin is formed during the ripening process. Nierenstein [1912], working with old Cheddar cheese, isolated from 6874 g. of cheese 82 g. of dried caseoglutin containing 14·52 % N. Osborne and Wakeman [1918] isolated from caseinogen prepared from milk a protein which was soluble in 50–70 % alcohol and very dilute acetic acid and gave a voluminous precipitate with potassium ferrocyanide. The tryptophan, Millon and biuret reactions were strongly positive. The phosphorus content was much less than that of caseinogen, and less arginine, histidine and lysine were found on hydrolysis. Anaphylactic reactions showed no genetic relationship between caseinogen and this alcohol-soluble protein. Grimmer and Wagenführ [1925] and Grimmer and Schutzler [1926] concluded from the analysis of the extract from Backstein cheese with 70 % alcohol that the caseoglutin obtained was a mixture of various substances, and that the composition was very variable.

Experimental.

In the course of the extraction of amino-acids from Cheddar cheese by the butyl alcohol method [Dakin, 1918] the presence of an alcohol-soluble protein was discovered.

To effect a preliminary separation of the amino-acids and lower polypeptides from caseinogen and the higher protein decomposition products in cheese 80 % alcohol was used. Powdered fat-free dried cheese was extracted with this reagent for 24 hours and the filtrate was evaporated at 40° under reduced pressure to obtain an aqueous residue for further extraction with butyl alcohol. It was found, however, that a bulky white precipitate was

formed as the ethyl alcohol was removed. The precipitate failed to dissolve on the addition of water to the aqueous residue but when ethyl alcohol was added until the concentration reached 80 % a clear solution was again obtained. A preliminary examination of the substance showed that it was a protein with a phosphorus content much less than that of caseinogen. A detailed investigation of its nature was therefore undertaken.

(*a*) *Protein from immature cheese.* An 18 weeks old Cheddar cheese was thoroughly grated and extracted with ether for 5 hours. The residue was dried in air for 24 hours at 55° to a dry, friable powder. 100 g. were suspended in 1 litre of 80 % alcohol and shaken at intervals over a period of 24 hours. The mixture was filtered and all the alcohol removed under reduced pressure at 40°. The bulky white precipitate, which was glutinous in parts, was filtered off and washed with cold water. On taking up in 80 % alcohol a slightly turbid solution was obtained. The turbidity was probably due to the denaturation of a portion of the protein since it had been previously noticed that the flocculent portion dissolved completely. On distilling this alcoholic solution at 40° under reduced pressure, filtering, washing the precipitate with water, and redissolving it in 80 % alcohol the amount of denaturation was found to be increased, with a lowering of the solubility.

The protein was precipitated from alcoholic solution with 2 volumes of ether, filtered and washed well with ether. This process was repeated once and the solid dried in air. In this way 0·8 g. of a pure white, friable powder was obtained.

Properties of the protein. The protein had the following properties.

(1) It was soluble in dilute alkali and re-precipitated from the solution on the gradual addition of dilute acid.

(2) An aqueous suspension dissolved on the addition of a little acetic acid.

(3) It was insoluble in water and absolute alcohol but completely soluble in 80 % alcohol. The colloidal suspension formed by adding water to this solution was flocculated by the addition of hydrochloric acid and could then be filtered.

(4) Sodium acetate gave no precipitate when added to the alcoholic solution. A fine white precipitate was produced by ether.

(5) Potassium ferrocyanide produced a bulky white precipitate from a solution in dilute acetic acid.

(6) The biuret, xanthoproteic and Millon reactions were positive and the Adamkiewicz and the Reichl reactions for tryptophan were also given.

(*b*) *Protein from mature cheese.* Investigations were made in order to see whether the same or a similar protein could be recovered from mature cheese. For this purpose a Cheddar cheese 16 months old, made from the same milk at the same time as the immature cheese, was used. 200 g. of fat-free, air-dried cheese were suspended for 24 hours in 1 litre of 80 % alcohol and then filtered. The filtrate was added to 3 volumes of water and the resulting precipitate separated by filtration. This light yellow precipitate failed to redissolve in

cold 80 % alcohol and became very glutinous. On warming to 40° some solution occurred, but separation into two layers took place on cooling, a slightly yellow, hard precipitate adhering to the bottom of the flask and a white, flocculent precipitate forming the top layer.

The white, flocculent precipitate was decanted and filtered, yielding a white, friable residue. Redissolving this residue in warm 80 % alcohol and adding the solution to two volumes of cold water produced a milky, colloidal suspension. On adding dilute alkali drop by drop a point was reached where the protein separated into flakes which filtered easily, leaving a clear filtrate of p_H 4·5. After drying in air at 30° the residue yielded 3·28 g. of a white, friable powder. The reactions were those of the protein isolated from the immature cheese.

The glutinous nature of the protein when mixed with alcohol appeared to be similar to that of the monocalcium paracaseinate of Van Slyke [1924] but its negligible ash content and insolubility in warm 5 % sodium chloride solution discounted this idea.

(*c*) *Protein from peptic digest of caseinogen.* In view of the presence in cheese of alcohol-soluble proteins an examination of the peptic digest of caseinogen for similar compounds was made.

100 g. of B.D.H. "Light White Casein" were extracted several times with 80 % alcohol at p_H 5·0, to remove any alcohol-soluble protein present, washed in ether and dried in air. Digestion with pepsin in 500 cc. of water acidified with hydrochloric acid was then carried out for 104 hours in the presence of chloroform. The volume was made up to 2½ litres with absolute alcohol, the mixture well shaken and the p_H adjusted to 5·0. Extraction was continued with occasional shaking for 15 hours and the solution filtered. When the alcohol was removed by distillation *in vacuo* at 40° a bulky precipitate separated. This was filtered, washed in ether, taken up three times in warm 80 % alcohol and finally dried. A yield of 0·25 g. of a white powder was obtained. The reactions were those of the first two proteins.

Composition of the three alcohol-soluble proteins. After drying *in vacuo* over sulphuric acid micro-combustions were carried out in duplicate on each protein to determine the carbon, hydrogen, nitrogen, sulphur, ash and moisture contents, by Dr Schoeller of Berlin. Table I shows the results of analysis.

Table I. *Composition of three alcohol-soluble proteins.*

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	52·43	52·04	52·51
H	7·98	7·55	7·26
N	14·36	14·15	14·38
S	0·93	0·83	1·02
Ash	0·003	Trace	0·004
Moisture	5·26	6·95	3·83

Phosphorus estimations. Colorimetric estimations by Briggs's [1922] method indicated a very low phosphorus content in all cases but by the method of

Benedict and Theis [1924], which avoids loss of phosphorus during incineration, appreciably higher values were obtained. For accurate determinations the method of Fiske and Subbarow [Hawk, 1927] and a dry ashing process were used. The method was tried out on a sample of caseinogen (prepared by the method of Van Slyke [1924]). Duplicate estimations gave values of 0·742 and 0·751 % for the phosphorus content of the moisture-free caseinogen. The values obtained for the phosphorus content of the three proteins were as follows:

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
P %	0·19	0·08	0·03

Discussion.

The composition of the three alcohol-soluble proteins, computed on a moisture- and ash-free basis, is shown in Table II, where the composition of caseinogen is also given for comparison.

Table II. *Composition of alcohol-soluble proteins and of caseinogen.*

Protein	Caseinogen	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	53·11	55·34	55·93	54·60
H	7·05	7·68	7·23	7·03
N	15·65	15·16	15·21	14·95
S	0·82	0·98	0·89	1·06
P	0·82	0·20	0·09	0·03
O	22·55	20·64	20·65	22·33

It is evident that the proteins differ considerably in composition from caseinogen and show some appreciable differences between themselves. Their main characteristic is their very low phosphorus content. Reference to Table I shows that the ash content of each is negligible, so that it may be assumed that the phosphorus present is in organic combination and is not an inorganic contamination.

Although the three proteins exhibit very similar characteristics and each has a very low phosphorus content they are not identical in composition. It is therefore important to decide whether the proteins from the cheese were initially present in the milk from which the cheese was made or whether they are decomposition products of the casein. The protein isolated by Osborne and Wakeman [1918] from caseinogen prepared from milk is very similar to the three proteins discussed in this paper and has a low phosphorus content—0·08 %. However, the caseinogen used by these authors was prepared from milk by precipitation with dilute hydrochloric acid, the product being purified by dissolving in alkali and precipitating with acid several times. If, therefore, we regard the alcohol-soluble protein finally isolated from the caseinogen as being initially present in the milk, it must be assumed that such treatment is without effect on the caseinogen molecule.

Rimington and Kay [1926] found that during the action of pepsin on

caseinogen no measurable amount of free phosphoric acid was liberated. Trypsin, however, converted rather more than 50 % of the phosphorus into free phosphoric acid in 2 months, while 1 % alkali at 37° liberated all the phosphorus as phosphoric acid within 24 hours. Rimington [1927] isolated from a tryptic digest of caseinogen a phosphopeptone which contained 50 % of the organic phosphorus. The molecule of this compound contained nine amino-acid linkages and was split off from caseinogen with great rapidity. Posternak [1927] isolated three similar compounds from a tryptic digest of caseinogen, but Rimington suggested that the presence of a phosphorus-free compound which followed the precipitation of the phosphopeptone rather closely was responsible for the variety of compounds obtained by this author.

Several workers have recently claimed that caseinogen itself is not homogeneous but is a mixture of several different proteins. Kondo [1923–25] and Linderstrøm-Lang [1925–27] were led to this conclusion by the fact that the solubility of caseinogen in dilute hydrochloric acid was found to be a function of the amount of undissolved caseinogen and that by fractional precipitation fractions could be obtained which differed considerably in P/N ratios. Linderstrøm-Lang [1927–29], using 60 % alcohol as a differential slovent, obtained from caseinogen several fractions with very different phosphorus contents. By re-combining the fractions it was possible to secure a mixture which exhibited the same properties (*e.g.* specific rotation, solubility in 60 % alcohol, solubility in hydrochloric acid, rate of rennet coagulation) as the original caseinogen.

Svedberg and co-workers [1930], by the application of the sedimentation velocity method and the equilibrium method [Svedberg, 1926, 1927], found that caseinogen prepared by Hammarsten's method consisted of several proteins of different molecular weights. By extracting caseinogen with 70 % alcohol acidified with hydrochloric acid they obtained a protein which they found to be a definite chemical entity, with a molecular weight of 375,000. Caseinogen prepared by the method of Van Slyke [1924] was also found to be heterogeneous.

There is thus some evidence for the conclusion that caseinogen in the free condition is a mixture of several different proteins. In any method of preparation, however, the use of dilute acid and alkali is essential for the purposes of preparation and purification and, having regard to the fact that caseinogen has been shown to be easily acted upon by alkali and by trypsin, resulting in the removal of the phosphorus, it appears very probable that the caseinogen molecule is so delicately constituted and the linkages so fragile that the treatment with acid and alkali to which it is subjected in the course of preparation is sufficient to rupture the molecule in several places, giving rise to a mixture of proteoses of lower molecular weight and differing phosphorus contents.

On these grounds, therefore, it is considered that the two proteins isolated from cheese are probably mixtures of several similar compounds, resulting

from breakdown of the casein either by the rennin, bacterial enzymes, or possibly the action of acid. The attempt to obtain a similar alcohol-soluble protein from the peptic digestion of caseinogen resulted, it is true, in a yield of only 0·25 g. from 100 g. of caseinogen. However, the caseinogen used was first extracted several times with 80 % alcohol and it is possible that this treatment removed nearly all the alcohol-soluble protein which was capable of being formed from the caseinogen and that treatment of the remainder with pepsin resulted mainly in the formation of other and lower degradation products.

SUMMARY.

Three alcohol-soluble proteins—two from Cheddar cheese and one from a peptic digest of caseinogen—have been isolated and their properties studied. The proteins differ considerably from caseinogen in composition and have a very low phosphorus content. Evidence is cited for the view that they are decomposition products of the caseinogen or the casein.

The author desires to thank Dr N. C. Wright for supplying a sample of caseinogen, Dr W. L. Davies for independent phosphorus determinations, Mr H. R. Whitehead for help with protein separations and Dr A. T. R. Mattick for his interest throughout this work and for many valuable suggestions.

REFERENCES.

Benecke and Schulze (1887). *Landwirtsch. Jahrb.* **16**, 317.
Benedict and Theis (1924). *J. Biol. Chem.* **61**, 63.
Bisegger (1907). Dissertation. Zurich.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Dakin (1918). *Biochem. J.* **12**, 290.
Grimmer and Schutzler (1926). *Milchwirtsch. Forsch.* **3**, 495.
—— and Wagenführ (1925). *Milchwirtsch. Forsch.* **2**, 183.
Hawk (1927). Practical physiological chemistry. (J. and R. Churchill, London.)
Kondo (1923–25). *Compt. Rend. Lab. Carlsberg*, **15**, No. 8.
Linderstrøm-Lang (1925–27). *Compt. Rend. Lab. Carlsberg*, **16**, No. 1, 48.
—— (1927–29). *Compt. Rend. Lab. Carlsberg*, **17**, No. 9.
Nierenstein (1912). *J. Agric. Sci.* **4**, 226.
Osborne and Wakeman (1918). *J. Biol. Chem.* **33**, 243.
Posternak (1927). *Compt. Rend. Soc. Phys. Hist. Geneva*, **44**, 1, 8.
Rimington (1927). *Biochem. J.* **21**, 202.
—— and Kay (1926). *Biochem. J.* **20**, 777.
Rose and Schulze (1884). *Landwirtsch. Jahrb.* **13**, 115.
Svedberg (1926). *Z. physiol. Chem.* **121**, 65.
—— (1927). *Z. physiol. Chem.* **127**, 51.
—— Carpenter and Carpenter (1930). *J. Amer. Chem. Soc.* **52**, 241.
Van Slyke (1924). *Chemical Age*, **32**, 163.
Weidemann (1882). *Landwirtsch. Jahrb.* **11**, 587.
Winterstein (1904). *Z. physiol. Chem.* **41**, 485.
—— and Thöni (1902). *Z. physiol. Chem.* **36**, 28.

CXVIII. THE FERRICYANIDE METHOD FOR THE DETERMINATION OF REDUCING SUGARS.

A MODIFICATION OF THE HAGEDORN-JENSEN-HANES TECHNIQUE.

By ALFRED CRESSWELL HULME and RAMJI NARAIN.

From the Botany School, Cambridge.

(*Received June 20th, 1931.*)

The modification of the Hagedorn and Jensen [1923] method for the estimation of reducing sugars introduced by Hanes [1929] has the advantage over copper reduction methods that much smaller amounts of sugar can be accurately estimated. Also, as Hanes showed, this method, unlike the copper reduction methods, gives results that are not appreciably affected by the oxygen tension in the solution being estimated. He employed the method for the estimation of glucose and maltose, the latter sugar giving a linear relation between the amount of sugar and amount of ferricyanide reduced while glucose did not give such a linear relation under Hanes's conditions.

Callow [1930] applied the Hanes method to the estimation of invert sugar, but the method has not been standardised for fructose.

In order to use this method for a study of carbohydrate metabolism in green leaves, employing small quantities of material, it was first necessary to investigate the sugar/ferricyanide relation for fructose and for varying mixtures of the reducing sugars (glucose, fructose and maltose). It was hoped also that it might be possible to find a set of conditions under which the sugar/ferricyanide relationship would be linear for all the reducing sugars. Under such conditions one might expect that the total reducing power would be the simple sum of the reducing values of the component sugars. This would enormously simplify the analysis of the extracts. From the results of Hanes and Callow it seemed likely that this might be attained by altering the time of boiling, or the volume or both. As the results of this paper show, this possibility has been realised by changing the volume alone.

Experimental.

Hanes's technique for the actual oxidation of the sugar by ferricyanide and the titration of the excess of ferricyanide with thiosulphate was carefully followed with the exception that the volume of sugar solution taken was 10 cc. in place of 5 cc.

The thiosulphate solution (approximately $N/75$) was standardised against standard potassium iodate by the method given by Hanes. It was found advisable to check the strength of this thiosulphate solution every 2 or 3 days, especially in warm weather. The chemicals used were of the A.R. quality as supplied by the British Drug Houses, Ltd.; the proportions used were exactly as given by Hanes.

Sugar samples.

Glucose. The glucose used was Dextrose A.R. (British Drug Houses), which had been dried *in vacuo* over phosphorus pentoxide to constant weight. The sugar so treated had an optical rotation of $[\alpha]_D^{20°} + 52{\cdot}78°$ in an 8 % solution; the calculated rotation was $[\alpha]_D^{20} + 52{\cdot}68°$. In order to check the dryness of the sample the true weight of glucose in a solution made by dissolving 5 g. of the above sample in distilled water and then making to 100 cc. was ascertained by making use of the density tables of Brown, Morris and Millar [1897]. By this method it was found that exactly 5 g. of glucose were present in this solution.

Invert sugar. This sugar was prepared from Saccharose A.R. (B.D.H.) which was ground to a fine powder and dried under diminished pressure over phosphorus pentoxide to constant weight, by the method of Lane and Eynon [1923]. A 10 % unneutralised solution had $[\alpha]_D^{20°} - 20{\cdot}62°$; the calculated value, allowing for the acid present, was $[\alpha]_D^{20°} - 20{\cdot}72°$. The rotation of the sucrose from which the invert sugar was prepared was $[\alpha]_D^{20°} + 66{\cdot}39°$ in a 2 % solution; calculated value $[\alpha]_D^{20°} + 66{\cdot}42°$.

Fructose. The fructose used was a sample of Kahlbaum's "Fructose kryst" which was ground to a fine powder and dried under diminished pressure at 60° over phosphorus pentoxide to constant weight. It had $[\alpha]_D^{27°} - 86{\cdot}40°$. This rotation is within the range of published values for fructose but is rather low compared with the theoretical value calculated from the formula suggested by Vosburgh [1920]. This fact tended to show that, although dried to constant weight under the conditions described, the fructose was not completely dry. This was proved by the fact that a solution containing 5 g. of the sample in 100 cc. of solution was found, from density determinations (as for glucose above), to contain actually only 4·930 g. of dry fructose. Hence apparent weights of fructose must be multiplied by 4·93/5·00 to give the true weight of dry fructose. This correction is embodied in all the results given for fructose; its application leads to the following optical rotation for the fructose sample used: $[\alpha]_D^{27°} - 87{\cdot}68°$, the theoretical value being $- 88{\cdot}08°$.

Maltose. The sample used was a Kahlbaum preparation of pure maltose monohydrate which had been recrystallised three times from 60 % alcohol and dried *in vacuo* as above to constant weight. The $[\alpha]_D^{20°}$ of an 8 % solution was $+ 130{\cdot}02°$; calculated value $+ 130{\cdot}07°$ [Parcus and Tollens—see Landolt, 1898].

The calculated values for the optical rotations of the sugars were obtained from the formulae given by Landolt [1898] unless otherwise stated.

Sugar solutions.

Two different methods of delivering the required amounts of sugar in solution were employed.

(*a*) Sugar solutions of 0·01 and 0·05 % were prepared and transferred to the boiling-tubes containing the ferricyanide from a 10 cc. burette (graduated to 1/50 cc. and fitted with a fine waxed jet) in the required quantities.

(*b*) Sugar solutions of 0·01, 0·02, 0·25 and 0·05 % were prepared and transferred to the boiling-tubes by means of accurate 2, 5 and 10 cc. pipettes.

In both cases the final volumes of sugar solution were made to 10 cc. with distilled water using a graduated pipette, when necessary. Water volume differences of 0·01 cc. had no measurable effect on the results.

Both methods (*a*) and (*b*) were used indiscriminately although in most cases duplicate estimations using the two methods were carried out, good agreement being obtained. In all cases the total number of duplicates was four and the maximum variation in cc. thiosulphate ($N/75$ approx.) among replicates was 0·03 cc.

Standardisation data.

Table I gives the mean values obtained for glucose, invert sugar, fructose and maltose. The actual readings of approximately $N/75$ thiosulphate have been converted to their $N/100$ equivalents for the sake of uniformity.

Table I.

Sugar mg.	cc. $N/100$ thiosulphate $-(WB-R)\times$ normality factor (mean of four estimations)			
	Glucose	Invert sugar	Fructose	Maltose
0·20	—	—	0·54	0·38
0·25	0·69	0·69	0·67	—
0·50	1·44	1·43	1·43	1·05
0·75	2·17	2·16	2·12	—
1·00	2·90	2·89	2·88	2·18
1·25	—	—	—	2·70
1·50	4·32	4·36	4·36	3·24
1·75	—	—	—	3·79
2·00	5·83	5·87	5·82	4·34
2·50	7·31	7·36	7·30	5·42
3·00	8·76	8·82	8·72	6·545
3·50	—	—	—	7·66

Above 3·0 mg. in the case of glucose, fructose and invert sugar and above 3·5 mg. in the case of maltose the results are not sufficiently accurate. This is due, no doubt, to the large percentage of the total ferricyanide used in the oxidation of the sugar in these cases.

The sugar-thiosulphate reaction.

From inspection of the glucose results it is clear that equal increments in sugar give approximately equal increments in thiosulphate, 0·25 mg. glucose corresponding on the average to 0·735 cc. thiosulphate. The observed value for an initial 0·25 mg. glucose is, however, 0·69 cc. so that although the

relation is approximately linear the line will not pass through the origin. The relationship between sugar and thiosulphate may therefore be put in the form

$$S = b\,(T + a),$$

a representing an amount in cc. thiosulphate to be added to the observed thiosulphate value, T, and b the conversion factor for thiosulphate to sugar, S. A similar relation holds for all the sugars under investigation. In future thiosulphate value T will be distinguished from the value $(T + a)$ by designating the former "observed thiosulphate value" and the latter "reducing value," T and a being expressed in terms of $N/100$ thiosulphate unless otherwise stated.

Fig. 1 shows the experimentally determined points for glucose and maltose (two separate series of determinations) and the lines corresponding to the calibration equation finally adopted, *i.e.* for glucose $S = 0{\cdot}340\ (T + 0{\cdot}05)$—see later.

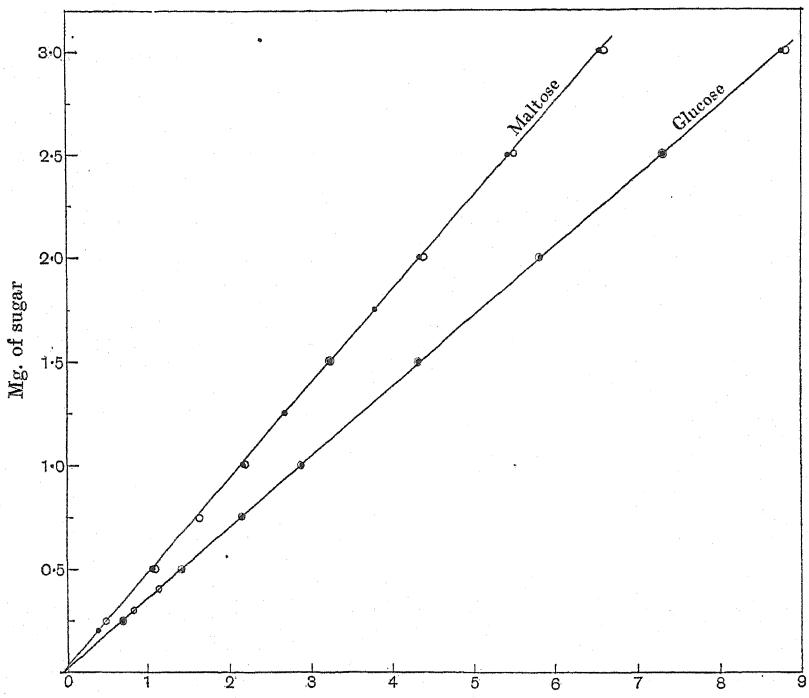


Fig. 1.

Values of a and b were obtained by the method of least squares taking the calculations to the third and fourth decimal place respectively. The values obtained are given in Table II.

As a measure of the accuracy with which these values fit the observations

Table II.

Sugar	a	b	S.D.$_c$
Glucose	0·041	0·3409	0·0065
Fructose	0·052	0·3410	0·0063
Invert sugar	0·059	0·3377	0·0046
Maltose	0·053	0·4545	0·0063

we give also, as S.D.$_c$, the standard deviation of calculated from observed values in mg. sugar.

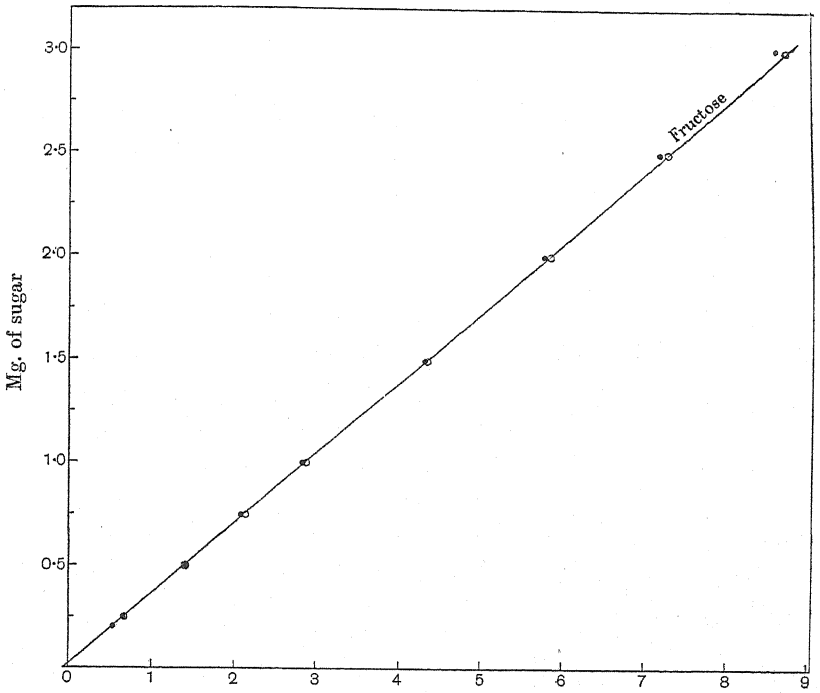


Fig. 2.

Consideration of factors a and b.

As it was impossible to read the thiosulphate burette, which was graduated in 0·02 cc. divisions, to a greater accuracy than 0·01 cc. the factors a and b can only have significance to the second and third decimal place respectively. Further, it is clear that the values of a are not significantly different for the different sugars and the mean value 0·05 can be used without loss of accuracy. Using this value of a and taking b to the third decimal place, the following rationalised values were obtained (Table III):

Table III.

Sugar	b	S.D.$_c$	S.D.$_e$
Glucose	0·340	0·0071	0·0033
Fructose	0·341	0·0067	0·0034
Invert sugar	0·338	0·0053	0·0028
Maltose	0·455	0·0077	0·0033

The figures show that by this rationalisation the standard deviation of calculated from observed is not appreciably increased. Hence the a factor of 0·05 with the b factors given in the above table are accepted as the standard values for the experimental conditions used. It will be seen that the fructose and glucose factors differ by less than 0·3 %, a fact of obvious advantage. The slightly lower value obtained for invert sugar will be discussed later.

The value S.D.$_e$ in Table III is the standard deviation in mg. sugar of the mean of four repeated determinations. It was calculated for each sugar from the expression:

$$\text{S.D.}_e = b\sqrt{\Sigma d/(f \times n)},$$

where d is the deviation in cc. $N/100$ thiosulphate from the mean of four repeated determinations, f is the total number of degrees of freedom (3 for each set of 4 repeated determinations), n the number of repeated determinations in each set (4), and b is the thiosulphate conversion factor for the sugar in question. Although the deviation from calculation (S.D.$_c$) is rather greater than the error due to successive estimations (S.D.$_e$), the scatter of the deviations about the sugar-thiosulphate lines calculated from the standard values of a and b does not suggest any significant departure from linearity. Moreover, it is clear that the linear relation fits the observations to within 0·5 % of the mean amount of sugar estimated (1·5 mg.).

Significance of the "a" factor.

A factor corresponding to the a factor (*i.e.* reduction curves not apparently passing through the origin) is common in copper reduction methods but had not been observed previously with the ferricyanide method; Hanes's curves, obtained for a sugar volume of 5 cc., passed through the origin. This fact, for 5 cc. sugar volumes, has been confirmed by the present authors so that the factors giving rise to a cannot be due to differences in personal technique. A confirmation of this fact is afforded by the agreement as to the value of a for 10 cc. sugar volumes obtained by the authors carrying out independent series of estimations. It might be expected that, using 15 cc. sugar volumes, a higher value for a would be obtained. Actually using such a volume it was impossible to obtain results sufficiently constant to be able to give any definite value to a under these conditions; values between 0·02 and 0·04 were obtained. This may be due to several causes—inequality of the initial stages of boiling, too high dilution of ferricyanide, *etc.*

The existence of factor a may mean (a) that some constant amount of sugar is destroyed before oxidation by ferricyanide, or (b) that a definite amount of re-oxidation of the ferrocyanide takes place during boiling, or (c) that auto-reduction of the ferricyanide occurs in water but not in the presence of sugar. This latter possibility seems hardly feasible in view of the identical water blank values obtained with 5, 10 and 15 cc. of water in the cold or with boiling for the standard time, while varying the volume of sugar solution does vary the value of a. Some evidence in favour of (b) is given by

the fact that Hanes found a somewhat low observed thiosulphate value when the sugar solutions were saturated with oxygen.

Thus it is difficult to find the theoretical significance of factor a, but from the point of view of actual sugar estimations it hardly warrants further investigation; indeed the factor may be neglected without introducing appreciable error so long as the amount of sugar to be estimated does not fall below 0·75 mg. Where a higher degree of accuracy is essential, and for amounts of sugar below 0·75 mg., the experimentally determined value of 0·05 cc. $N/100$ thiosulphate is quite satisfactory. To avoid error the factor is best applied by adding 0·05 to the water blank value, expressed as $N/100$ thiosulphate, when the requisite b factor may be applied directly.

Mixtures of sugars.

From the linearity of the relation between sugar and thiosulphate it is clear that a unit amount of sugar gives a constant reduction of ferricyanide in all parts of the scale investigated. It is essential to ascertain whether, in mixtures of sugars, one unit of each sugar will bring about a constant amount of ferricyanide reduction quite independent of the ferricyanide reduction caused by the other sugars. This was tested by comparing the observed thiosulphate value for mixtures of sugars with the simple sum of the calculated values for the component sugars. The results obtained are summarised in Table IV.

Table IV.

Sugar mixture (mg. sugar)				cc. $N/100$ thiosulphate	
Glucose	Invert sugar	Fructose	Maltose	Observed	Calculated
0·50	—	—	0·50	2·56	2·52
1·00	—	—	1·00	5·12	5·08
0·80	2·00	—	1·00	10·47	10·45
0·64	3·20	—	—	11·30	11·30
—	1·25	—	1·25	6·41	6·40
0·424	—	1·972	—	6·94	6·97
1·060	—	1·233	—	6·68	6·68
0·530	—	1·972	—	7·27	7·29
1·500	—	1·500	—	8·74	8·76

From these results it is clear that each sugar behaves towards ferricyanide as if the others were absent. However, the case of invert sugar considered as a mixture of equal parts of glucose and fructose is an apparent exception to this generalisation since the b factor, 0·338, is 0·0025 lower than the mean for glucose and fructose, 0·3405. "Synthetic" invert sugar (equal amounts of glucose and fructose) gives the expected factor as can be seen from the results for such an "invert sugar" solution given in Table IV. This suggests that the reducing power of invert sugar (as prepared by hydrochloric acid inversion of sucrose) with respect to ferricyanide is slightly higher than the expected additive reducing power of glucose and fructose. To suggest a reason for this would be pure speculation, although one obvious but unexpected possibility is that the acid present when the glucose and fructose are being formed from

sucrose might in some way slightly affect the reducing power of the two sugars. However, on treatment of a mixture of equal parts of glucose and fructose with 2·5 % hydrochloric acid under the conditions given by Hinton and Macara [1927] for the preparation of invert sugar from sucrose, the ferricyanide reducing power of the glucose and fructose was found not to have been affected in the slightest, the theoretical reducing value being found using the glucose-fructose *b* factors; see Table V.

Table V.

Glucose + fructose (mg.) (equal amounts)	Reducing value in cc. *N*/100 thiosulphate		
	Observed	Calculated for *b* factor for glucose + fructose	Calculated for *b* factor for invert sugar
2·134	6·23	6·22	6·27

Thus the possibility of hydrochloric acid affecting the reducing power of glucose and fructose under the conditions of sucrose inversion employed is ruled out. That the Hinton and Macara method for sucrose hydrolysis gives similar results to the Lane and Eynon method used in the invert sugar standardisation will be seen from Table VII, since the use of the *b* factor obtained for invert sugar prepared by the latter method gives agreement between actual and observed amounts of invert sugar prepared by the former method.

In addition to investigating the effect of one reducing sugar on another, the effect of the presence of sucrose was also investigated, since in copper reduction methods the presence of this sugar affects the reducing value obtained for reducing sugars. As will be seen from the results given in Table VI, the ferricyanide method is not appreciably affected by the presence of sucrose.

Table VI[1].

	Sucrose (mg.)	cc. *N*/100 thiosulphate
Glucose approx. 1·0 mg. (constant)	0·5	2·92
	1·0	2·92
	2·0	2·92
	4·0	2·94
Invert sugar approx. 1·0 mg. (constant)	0·5	2·94
	2·5	2·95

Fructose and maltose results are also unaffected by the presence of sucrose.

The fact that the total reducing value of a mixture of sugars, as estimated by the modified ferricyanide method described in this paper, is the simple sum of the reducing values of the component sugars and is not affected by the concentration of sucrose should give the method a great advantage over copper reduction methods in the estimation of the individual sugars present

[1] Note added July 29th. The authors have since found that sucrose alone does not cause any appreciable reduction of ferricyanide under the conditions of the modified Hanes estimation; this confirms the advantage of this method over copper reduction methods.

in, for example, leaf extracts. In the simplest case, the estimation of sucrose in a mixture of sugars, the increase in the reducing value following inversion may be converted directly into mg. invert sugar, using the appropriate factor (0·338). The invert sugar used for standardisation was prepared by hydrochloric acid inversion in the cold. In order to test whether the use of other methods of inversion would affect the ferricyanide reducing value, estimations of sucrose were carried out (1) by the citric acid inversion method and (2) by the hydrochloric acid inversion for 12 minutes at 60° as suggested by Hinton and Macara [1927].

(1) Inversion was carried out by 10 % citric acid in a boiling water-bath for 15 minutes, cooling and neutralising the solution with 5*N* NaOH; the requisite amount of NaOH was previously found on a separate amount of sugar-citric acid solution, using bromothymol blue indicator. The solution was then made to a definite volume and an aliquot taken for a reducing value estimation. It was found unsatisfactory to take 10 cc. of the neutralised solution for this estimation, for if this was done, the citrate present interfered with the precipitation of the potassium zinc ferrocyanide causing a shifting, indefinite end point, especially in the case of small amounts of sugar. It was found possible, however, to overcome this difficulty by taking only 5 cc. of inversion liquid and 5 cc. of distilled water, and then adding to the solution after the boiling with ferricyanide and cooling, in addition to the usual solutions, 1 cc. of 15 % zinc sulphate solution. The smaller amount of citrate present combined with the extra excess of zinc sulphate, and allowed of complete precipitation of the potassium zinc ferrocyanide, so that the thiosulphate titration end point was once more fixed and definite. The results obtained are given in Table VII.

(2) By the Hinton and Macara method, inversion was carried out by approximately 2·5 % hydrochloric acid in a water-bath at 60° for 12 minutes, agitating the liquid during the first 3 minutes of inversion. The solution was then neutralised with 5*N* NaOH and made to a definite volume; aliquots were taken for the determination of the reducing value. In both the citric and hydrochloric acid methods of inversion, control experiments were carried out, the sugar solution being replaced by distilled water. These controls were treated in a manner identical with that used for the sugar solutions and

Table VII.

	mg. sugar		% deviation observed from present
	Present	Observed	
Citric acid hydrolysis:			
Sucrose (as invert sugar)	0·263	0·267	1·5
,,	1·315	1·324	0·7
,,	2·632	2·670	1·4
HCl hydrolysis:			
Sucrose (as invert sugar)	1·894	1·894	Nil
,,	1·067	2·140	0·3
Glucose, fructose (equal amounts)	1·067		

the water blank (W.B.) value used for the reducing value determinations on the invert sugar solutions was obtained from these controls.

In order to confirm the validity of the hydrochloric acid method of inversion in sugar mixture determinations, an inversion was also carried out in the presence of equal amounts of glucose and fructose.

The results of the above determinations are given in Table VII.

Thus for estimation of sucrose as invert sugar by the ferricyanide reduction method inversion by 2·5 % hydrochloric acid for 12 minutes at 60° gives accurate results, while citric acid inversion gives results which are appreciably high. These high results are probably partly due to the effect of the citrate on the potassium zinc ferrocyanide. The presence of hydrochloric acid does not affect the reducing power of the other reducing sugars under the conditions of sucrose inversion employed. The hydrochloric acid method has, therefore, been adopted by the authors, although invertase inversion methods are being investigated to ascertain whether invert sugar prepared by this latter method gives a similar b factor to the one obtained on acid inversion.

For the hydrolysis of maltose in the presence of fructose or sucrose no really satisfactory method of acid hydrolysis is as yet available and investigations on this point and on the use of maltase are being carried out, especially in relation to sugar estimations in plant extracts.

For mixtures of glucose and fructose the modified Hanes method combined with iodimetric estimation gives easily calculated and accurate results.

The application of these two methods for the estimation of the individual sugars in simple mixtures of glucose, fructose and maltose and in leaf extracts is under investigation.

Summary.

1. A modification of the Hanes technique for the estimation of reducing sugars by oxidation with ferricyanide is described. This modification is shown to give a linear relation between the amount of sugar and the amount of ferricyanide reduced over a range of 0·2 to 3·0 mg. in the case of glucose, fructose and invert sugar, and of 0·2 to 3·5 mg. in the case of maltose. By statistical treatment the linear relation is found to be accurate to 0·0067 mg. sugar (mean value for the four sugars), *i.e.* 0·5 % of the mean amount of the sugars estimated.

2. Expressing the relation between amount of sugar and the amount of ferricyanide reduced in terms of $N/100$ thiosulphate, the following relation is found:

$$\text{Sugar} = b\,(\text{observed thiosulphate} + a),$$

a having the value 0·05 cc. $N/100$ thiosulphate for all the sugars investigated; factor b is shown to be, for glucose 0·340, for fructose 0·341, for invert sugar (prepared by HCl inversion of sucrose) 0·338 and for maltose 0·455.

3. In a mixture of sugars the ferricyanide reducing power of one sugar is shown to be unaffected by the presence of the others. The presence of

sucrose does not affect the reducing value of the other sugars present in a mixture.

4. Invert sugar, prepared by acid hydrolysis of sucrose, has a slightly higher reducing value than a synthetic mixture composed of equal amounts of glucose and fructose.

5. For the estimation of sucrose, inversion by 2·5 % hydrochloric acid for 12 minutes at 60° (after Hinton and Macara) followed by determination of ferricyanide reducing power is found to give accurate results, while citric acid inversion gives results which are too high.

6. The application of the ferricyanide reduction method to the estimation of individual sugars in plant extracts is discussed.

The authors' best thanks are due to Dr E. J. Maskell for his continued advice and helpful criticism. To Dr F. F. Blackman we are indebted for interest and encouragement.

One of the authors (A. C. H.) is also indebted to the Department of Scientific and Industrial Research for a maintenance grant which has made the work possible.

REFERENCES.

Brown, Morris and Millar (1897). *J. Chem. Soc.* **71**, 72.
Callow (1930). *Biochem. J.* **24**, 57.
Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
Hanes (1929). *Biochem. J.* **23**, 99.
Hinton and Macara (1927). *Analyst* **51**, 668.
Landolt (1898). Das optische Drehungsvermögen, 1 Auflage.
Lane and Eynon (1923). *J. Soc. Chem. Ind.* **42**, 32.
Vosburgh (1920). *J. Amer. Chem. Soc.* **42**, 1696.

CXIX. THE CARBOHYDRATE COMPLEX OF THE SERUM PROTEINS.

II. IMPROVED METHOD FOR ISOLATION AND RE-DETERMINATION OF STRUCTURE. ISOLATION OF GLUCOSAMINODIMANNOSE FROM PROTEINS OF OX BLOOD.

By CLAUDE RIMINGTON.

From the Biochemical Department of the Chemical Laboratories, The Wool Industries Research Association, Torridon, Headingley, Leeds.

(*Received May 16th, 1931.*)

In a previous communication [Rimington, 1929] the isolation was reported of a nitrogen-containing polysaccharide from both the albumin and globulin of horse's blood-serum. The yield from each protein was about 2 %. It was also shown that this non-reducing complex gave rise, upon acid hydrolysis, to glucosamine and a hexose which was identified as mannose. On the basis of analytical figures, more particularly the nitrogen content of the isolated complex, it was concluded that the simple structure consisted of 1 molecule of glucosamine associated with 1 molecule of mannose and a structural formula was proposed which would account both for the lack of reducing property and for the liberation of nitrogen from the amino-group when acted upon by nitrous acid.

Shortly after this paper was published, a communication appeared from Levene and Mori [1929] who claimed to have isolated a carbohydrate complex from ovalbumin and ovomucoid in which 1 molecule of glucosamine was associated with 2 molecules of mannose. From molecular weight determinations by the diffusion method, Levene and Rothen [1929] came to the conclusion that the complex was a polymeride consisting of 4 units of the trisaccharide, *i.e.* 12 hexose residues in all. These workers found that relatively prolonged hydrolysis by baryta was necessary to free the carbohydrate completely from accompanying protein material.

Since the carbohydrate isolated from egg-protein by Levene and Mori was in many respects similar to that obtained from serum, it was thought to be desirable to reinvestigate the conditions under which the latter could be obtained in pure form and to establish with certainty its ultimate di- or trisaccharide nature.

It was found that by further prolonged hydrolysis of the preparations previously reported upon, some nitrogenous material was eliminated, leaving

a substance with a nitrogen content which became constant at 2·7 % agreeing with that required by a trisaccharide structure. The impurity present in the original preparations was only removed with difficulty and by the application of relatively vigorous methods. It proved to consist largely of histidine. By comparison of the analytical figures for histidine with those required by the di- and tri-saccharide formulae it will readily be appreciated that its presence in small quantity might be overlooked. The pure carbohydrate has now been

	C	H	N
Histidine	46·42	5·85	27·10
Disaccharide (glucosamine + 1 mannose)	42·21	6·79	4·10
Trisaccharide (glucosamine + 2 mannose)	42·94	6·56	2·78

obtained from the serum proteins of the horse and ox. In each case the composition indicates a trisaccharide nature and as far as it is possible to tell, the preparations appear to be identical.

Levene and Mori found that from ovomucoid as much as 5 % of carbohydrate could be isolated and concluded, therefore, that this protein was the source of the material isolated from crystallised ovalbumin preparations. Although the carbohydrate content of egg-white appeared to decrease on crystallisation of the protein it is difficult to believe that a three times recrystallised preparation could have contained as much as 5 % of associated ovomucoid, the figure demanded by the quantities of carbohydrate isolated from each individual protein (5·1 % from ovomucoid, 0·26 % from thrice recrystallised ovalbumin).

Sørensen [1930] has shown that egg-albumin dissociates to some extent in solution, so that by successive recrystallisation fractions are continually being left behind in the mother-liquors which differ from the crystalline phase by possessing a relatively higher solubility; these fractions also show differences in chemical composition. It might well be that, by this process, carbohydrate-rich fractions are eliminated from the bulk of the protein, a hypothesis which would explain the decreasing carbohydrate content observed by Levene and Mori, of repeatedly recrystallised egg-albumin more readily than the supposition that only ovomucoid contains carbohydrate, the albumin being free from carbohydrate.

In the case of blood it is known that there is present in the serum a protein to which the name seromucoid has been given [Zanetti, 1897, 1903; Bywaters, 1909]. Although present to the extent of only 0·1 to 0·5 g. per litre of blood, seromucoid has been credited with a high carbohydrate content, 20 % or more, which if true, would mean that the sugar so found, accounted for as much as 10 % of the total protein-sugar of the blood.

An investigation of this protein is in progress and will be published shortly. It appears to contain a carbohydrate complex similar to that present in the albumin and globulin and in quantity approaching 25 % (determined colorimetrically by comparison with glucose and recalculated in terms of glucosaminodimannose).

For reasons similar to those outlined above in the case of egg-proteins, it is considered unlikely that the carbohydrate isolated from serum-albumin and globulin preparations was in reality derived from admixed seromucoid. The quantity obtained, about 2 % of the weight of the protein, was too great to render this likely, moreover evidence is presented below which proves all but conclusively that, in the case of horse serum-globulin, the carbohydrate enters into the protein molecule as a true constituent to the extent of approximately 3·7 %.. Final solution of the question may be deferred until the identity of the seromucoid carbohydrate has been established. It is noteworthy, however, that Lustig and Haas [1931] have recently investigated the carbohydrate content of blood-albumin and globulin preparations obtained by an elaborate series of fractionations and have recorded values which strongly support the view that carbohydrate enters into each of these proteins as an integral constituent.

That the carbohydrate complexes isolated from the proteins of horse and ox bloods appear to be identical does not, at first sight, appear to support the view put forward in the first paper of this series [Rimington, 1929] that the polysaccharide may play an active part in immunity reactions. This point is, however, under consideration and may be the subject of further communications. That what is apparently the same carbohydrate appears in so many different situations and species is undoubtedly a remarkable fact.

With regard to the structure of the trisaccharide complex there seems to be no reason why the argument originally put forward in support of a betaine-like linkage involving the nitrogen atom of the glucosamine molecule should not be equally applicable to the case of three hexose residues united together. Only exhaustive methylation and hydrolysis would show the point of attachment of the constituents. The slow liberation of nitrogen from the amino-group indicates however, as formerly, a union similar to that existing in glucosamine methyl glucoside. Karrer [1930] has put forward a similar view to account, on structural lines, for the known properties of chitosan. More recently Bertho *et al.* [1931] have discussed this question in relation to their work upon synthetic N-acyl derivatives of glucosamine and suggest that proximity of the $—NH_2$ to the —CHO group may alone be sufficient to cause abnormality in the reactions of the latter. The lack of activity of the aldehydic group towards the usual reagents would be interpreted by these authors as due to steric hindrance rather than to the existence of a true betaine grouping.

As to how the carbohydrate complex is united to the protein, no suggestion is at present ventured. It is however certain that, whatever the linkage, it is of a type that is markedly resistant to prolonged alkaline hydrolysis. Different proteins appear to vary in this respect, since in the present case 30 to 36 hours' boiling with 10 volumes of 10 % baryta was found necessary to eliminate all accompanying material from the blood-serum carbohydrate, whilst Levene

and Mori found that pure material could be obtained from the various egg-proteins after only 7 or 8 hours' boiling with the same reagent.

Unfortunately, appreciable destruction of the carbohydrate occurs during the more extended periods of hydrolysis with consequently lower yields, and no way has been found up to the present of obviating this loss of material.

Experimental.

As a result of previous work the conclusion had been drawn that the carbohydrate complex obtainable from serum-albumin and globulin was a disaccharide structure (possibly polymerised) containing one molecule of glucosamine and one of mannose and having therefore a nitrogen content of 4·1 %. Preparations isolated after 3 hours' hydrolysis of the protein by 10 % baryta appeared to approximate fairly closely in composition to this figure, provided glacial acetic acid had been used in the final stages as a precipitating reagent. If this treatment, which involved considerable loss of material, was omitted, nitrogen contents prevailed of 4·5 % and upwards. As a result of the investigations detailed below it appears that the carbohydrate is in reality a polymeride of a trisaccharide containing 2 molecules of mannose associated with every one of glucosamine, and thus possessing a nitrogen content when pure of 2·78 %. Certain modifications have been made in the method of isolation, details of which will be found below.

Preparation of carbohydrate from mixed proteins of horse-serum.

100 g. mixed albumin and globulin were boiled under reflux with 1 litre of 10 % barium hydroxide for 8 hours. (This proved to be too short a time; in later experiments 30–36 hours' hydrolysis was allowed.) To the filtered solution, sufficient 50 % sulphuric acid was added to render the reaction faintly acid to litmus and the barium sulphate removed by filtration and washed. 150 cc. of saturated basic lead acetate were added and then saturated baryta until maximal precipitation was attained. After removal of this precipitate on the Büchner funnel the filtrate was found still to give a faintly positive Molisch test. A further 75 cc. of basic acetate was therefore added and baryta as before. After this precipitation the solution was carbohydrate-free. The lead-barium precipitate was ground into a thin cream with water and sufficient 33 % acetic acid added to make the mixture faintly acid to litmus. The bulk of the precipitate dissolved leaving an orange-yellow material. If this gives a positive Molisch reaction, positive addition of acetic acid is cautiously continued until all the carbohydrate is in solution. The undissolved residue is filtered off and to the yellow-brown solution (allowing the acid solution to stand for 12–24 hours is recommended, since during this time a deposit of dark orange material settles out which can be filtered off and discarded) is added 3·0 cc. of basic lead acetate followed by baryta as before. The carbohydrate-lead compound is soluble in excess of baryta, so care should

be taken during the addition. After repetition at least three times of the solution in acetic acid and reprecipitation by baryta, the final lead-baryta precipitate is suspended in water and decomposed by a stream of carbon dioxide at 50–55°. Re-suspension of the lead carbonate and treatment with CO_2 is repeated until all the carbohydrate has passed into solution. To the combined liquids, a moderate excess of Hopkins's reagent (10 % $HgSO_4$ in 5 % H_2SO_4) is added and the mixture allowed to stand for several days. It is important that the total concentration of acid should not exceed 5 % whilst this precipitation is going on. As will be described later the material removed by mercuric sulphate has been shown to consist largely of histidine, and Calvery [1929] has shown that the histidine-mercury compound is soluble in sulphuric acid exceeding 5 % in strength.

After filtration lead and mercury are removed by hydrogen sulphide and the sulphuric acid by baryta. The solution is then concentrated *in vacuo* to a small bulk and the final elimination of SO_4 or Ba ions effected at this stage. Further concentration is performed in a vacuum desiccator over sulphuric acid, and to the syrup remaining is added about 20 cc. of dry methyl alcohol followed by an equal volume of ether. The precipitated carbohydrate is centrifuged, reprecipitated once or twice from concentrated watery solution and finally dehydrated by absolute alcohol and ether. Should the nitrogen content prove to be high, a further hydrolysis for 6–10 hours by 10 % baryta is necessary, followed by isolation as above, but in this case the basic lead acetate-baryta precipitation may be omitted.

Levene and Mori observed that pure material, from which all that was precipitable by mercuric sulphate had been removed, yielded nevertheless an additional precipitate with this reagent after further hydrolysis had been performed.

Precisely similar findings were encountered in the present case, which might be taken to indicate that the carboxyl group of the histidine, chief constituent of the mercury precipitate, is involved in some way in the union with the carbohydrate. Too great weight is not to be attached to this interpretation; it is put forward rather as a tentative suggestion as to how the complex may be united to the protein.

A yield of 3·5 g. of carbohydrate having a nitrogen content of 5·23 % was obtained. Since it was obviously still impure a further 8 hours' hydrolysis by baryta was performed and the isolation repeated. Yield 0·9 g. having 3·48 % of N; after 10 hours' more hydrolysis, 0·38 g. of pure material was obtained with 2·79 % of N.

In order to demonstrate that the nitrogen content remained constant at this figure and that the material was therefore pure, a further hydrolysis for 10 hours in 10 % baryta was carried out and 0·18 g. of material recovered with 2·66 % of N.

The steps in this purification, the yields and analytical data are summarised on p. 1067.

100 g. mixed proteins of horse-serum.

Hydrolysis (hr.)	Yield g.	Ash-free C	Ash-free H	Ash-free N*	Reaction with diazobenzenesulphonic acid	$HgSO_4$ ppt.
8	3·5	44·30	6·56	5·23	+	+ +
8 more	0·9	41·50	7·05	3·48	+	+
10 ,,	0·3	41·58	6·39	2·79	−	−
10 ,,	0·1	41·91	6·31	2·66	−	−
Calculated for $C_{18}H_{33}O_{15}N$		42·94	6·56	2·78		

* Micro-Kjeldahl by Mr A. R. Colwell, Cambridge. It was found that determinations on the same material by micro-Dumas gave constantly high results. C and H micro-determination by Dr Schoeller, Berlin.

Since histidine is the most tenaciously adhering impurity, applying the diazo-reaction with diazotised sulphanilic acid affords an indication of the progress of purification. The pure carbohydrate gives no reaction, neither does any precipitate form when mercuric sulphate reagent is added to its solutions after a further hydrolysis. Either test may be used to check the purity of material during the course of isolation.

Properties of the pure carbohydrate.

As precipitated from watery solution by alcohol and ether, the carbohydrate complex is obtained as a hygroscopic, creamy-white amorphous material. Its solutions are pale straw-coloured, viscous when concentrated, and show no measurable optical activity. They do not reduce Fehling's solution until after acid hydrolysis. The absence of optical activity may be due to racemisation during the prolonged hydrolysis. In the Van Slyke apparatus nitrogen is slowly evolved indicating the presence of a free amino-group as discussed previously. After acid hydrolysis no sugar except mannose and glucosamine could be detected.

Isolation from mixed proteins of ox-serum.

100 g. of the heat-coagulated mixed proteins from ox-serum[1] was boiled under reflux with 10 times the quantity of 10 % baryta. The hydrolysis was allowed to proceed uninterruptedly for 30 hours, after which the process of isolation was gone through precisely as described for the horse-serum carbohydrate. A yield of 1·1 g. was obtained. The N content was 3·72 %, thus indicating that nitrogenous impurities were still present. The whole of the material was therefore again hydrolysed with 10 % baryta for 12 hours. In carrying out the isolation the entire process was repeated without omission of any of the stages. Yield 0·48 g. having N 2·70 %. The full analyses were as follows:

	Ash-free C	Ash-free H	Ash-free N	Yield g.
Initial 30 hours' hydrolysis	42·94	6·31	3·72	1·1
After further 12 hours' hydrolysis	44·65	6·53	2·70	0·45
Calculated for $C_{18}H_{33}O_{15}N$	42·94	6·56	2·78	—

In general properties, the material was similar to that isolated from horse-serum proteins.

[1] For the preparations of ox-globulin and albumin I am indebted to Messrs Boots Pure Drug Co., Ltd., Nottingham, who kindly placed this quantity at my disposal.

Detection of histidine among the impurities present in the crude preparation.

As mentioned previously, it was found that crude preparations of the carbohydrate still contained nitrogenous impurities which were not precipitable by Hopkins's reagent, but which could be liberated by further boiling of the complex with baryta and were then removable as their mercury salts.

A small quantity of the mercury precipitate was suspended in water and decomposed by prolonged saturation with hydrogen sulphide gas. After removal of the sulphide, which showed a marked tendency towards the colloidal form, a portion of the solution was subjected to the following tests.

Biuret reaction	Very faintly positive; pink
Ninhydrin reaction	Intense
Millon reaction	Negative
Glyoxylic reaction	Positive, but atypical
Molisch reaction	Faintly positive, but accompanied by a dark brown colour.
Diazo reaction (sulphanilic acid)	Intense, deep red
Diazo reaction (sulphanilic acid) as modified by Totani	Clear golden yellow after addition of hydrogen peroxide
Phosphotungstic acid	Immediate precipitate, soluble on warming but reappearing as the solution cooled

The remainder was purified through the phosphotungstate and then gave all the typical reactions of histidine; the quantity present, however, was not great. The filtrate from the histidine phosphotungstate no longer gave any reaction with ninhydrin. It would appear from the above that histidine constitutes the major part of the impurity in crude carbohydrate preparations. It must be in some way combined with the polysaccharide since acid mercuric sulphate fails to precipitate it from solution.

Determination of the carbohydrate content of the serum-proteins by colorimetric methods.

Since isolation of a substance from a complex mixture is invariably attended by some degree of unavoidable loss, an attempt was made to determine the true carbohydrate content of the serum-proteins by an indirect means.

Methods available for the determination of carbohydrate materials fall into two classes, those depending upon the reducing property of the free aldehydic group and colorimetric methods. The former are inapplicable in this case since the conditions under which the protein gives rise to reducing sugars (acid hydrolysis) are also those favourable to secondary reactions whereby carbohydrate and amino compounds combine to form "humin" [see Gortner, 1916].

A colorimetric method has been proposed by Dische and Popper [1926] based upon the interaction between carbohydrates and indole in the presence of 70 % (by volume) sulphuric acid as condensing agent. The colour value is not strictly proportional to the quantity of sugar but this deviation is easily

corrected for[1]; moreover Dische and Popper showed that the same value was given by sugars whether in the free or combined state, thus starch and glycogen could be matched directly against glucose. Mannose gives only about 65–70 % of the colour of glucose whilst glucosamine does not react at all. The method is applicable to the determination of the combined carbohydrate in proteins and in the present writer's hands has proved most serviceable. A very similar method is that proposed by Tillmans and Phillipi [1929] who employ orcinol together with 60 % (by volume) sulphuric acid. This was the method employed by Lustig and Haas [1931] in their examination of serum-globulin subfractions. Mannose reacts quantitatively like glucose, but the amino-sugars fail to give any colour, as in the indole method.

From a comparison of the two methods one would expect that the pure glucosaminodimannose complex would have a higher glucose equivalent by the latter than by the former, which is indeed the case. Further support is thus offered to the structure already assigned to the complex. In the investigations with proteins, Dische and Popper's method was more frequently employed although both this and Tillman and Phillipi's orcinol method yielded the same result (see below).

Pure glucosaminodimannose (N, 2·70 %).

1·675 mg. was dissolved in 5 cc. of water and 1 cc. aliquots compared with a 0·02 % glucose solution.

By Dische and Popper's method 1·675 mg. polysaccharide≡0·6695 mg. glucose. Hence 39·96 % of the colour of glucose.

By Tillman and Phillipi's method 1·675 mg. polysaccharide≡1·099 mg. glucose. Hence 65·60 % of the colour of glucose.

Assuming mannose to react quantitatively, theory would require 66·6 %.

Ox-serum globulin*:

Dissolved 0·1024 g. in 5 cc. of 0·05 *N* sodium hydroxide.

By micro-Kjeldahl N = 11·825 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·468 % as glucose by Dische and Popper's method, *i.e.* 3·67 % of the protein when calculated as glucosaminodimannose.

Total carbohydrate (on 1 cc. aliquot) = 2·279 % as glucose by Tillman and Phillipi's method, *i.e.* 3·47 % of the protein when calculated as glucosaminodimannose.

* For this preparation I am indebted to Messrs Boots Pure Drug Co., Ltd., of Nottingham.

The total nitrogen was taken as 15·85 % in calculating the carbohydrate present.

The agreement between the two methods is satisfactory. The preparation of horse-serum globulin utilised by the present writer in the experiments culminating in the isolation, for the first time, of the carbohydrate complex was now examined. This protein sample had been very thoroughly purified [see Rimington, 1929], however, its carbohydrate content was found to be very similar to that of the ox-globulin above, and moreover this figure was found to suffer no decrease after thorough washing of the suspended protein particles, a treatment which would, undoubtedly, have removed any seromucoid still adhering.

[1] An empirically determined correction curve was employed, not the formula of Dische and Popper.

Horse-serum globulin, highly purified preparation.

Dissolved 0·1005 g. in 5 cc. of 0·05 *N* sodium hydroxide.

By micro-Kjeldahl N = 13·873 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·3 % as glucose, *i.e.* 3·74 % of the protein when calculated as glucosaminodimannose.

A further sample of about 0·1 g. of protein was placed in a stoppered centrifuge tube with 30 cc. of distilled water and subjected to vigorous mechanical shaking for 6 hours. After centrifuging and washing it was dissolved in dilute alkali as above and the carbohydrate content redetermined.

By micro-Kjeldahl N = 14·755 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·73 % as glucose, *i.e.* 4·32 % of the protein when calculated as glucosaminodimannose.

It seemed of interest to compare the carbohydrate content of the protein as determined colorimetrically with the actual yield obtainable by isolation. From the above figures it is clear that this sample of horse-serum globulin contained about 3·8 % of glucosaminodimannose. After an initial 3½ hours' hydrolysis there had been isolated 3·91 % of material having a nitrogen content of 4·92 %. As it is now known that the substance when pure has 2·78 % N, and that the major portion of the impurity is in all probability histidine (N, 27·10 %) it is permissible to deduct 9 % by weight corresponding to this amino-acid and to assume that the remaining material would be pure glucosaminodimannose. The corrected yield works out at 3·6 % in very good agreement with the content of 3·8 % determined colorimetrically. Of course, much loss is incurred if the crude material is boiled for any considerable length of time with baryta in order to effect further purification, so that yields of pure material as high as this are not directly attainable. In the previous investigation the yield had dropped to a little over 2 % by the time the nitrogen content was reduced to 4 %.

It seems quite certain that the carbohydrate content of the serum-proteins is considerably higher than has previously been supposed.

Summary.

1. A reinvestigation of the conditions under which the carbohydrate complex of the serum-proteins can be isolated in the pure state has led to modifications in the scheme of separation originally proposed.

2. It also appears that the carbohydrate is composed of an association of one molecule of glucosamine with two of mannose, being thus similar to that obtainable from egg-proteins.

3. The impurity accompanying the original preparations has been shown to consist largely of histidine. This amino-acid appeared to be united chemically to the complex.

4. Glucosaminodimannose has been isolated from the mixed serum-proteins of both horse- and ox-blood.

5. Colorimetric methods have been used to determine the carbohydrate

content of proteins. By reference to the colour value of the pure complex, such figures are expressible directly as glucosaminodimannose. The values obtained by two different methods are in agreement.

6. Serum-globulin appears to contain approximately 3·7 % of carbohydrate.

7. This figure is not decreased by thorough washing of the coagulated protein and is taken therefore to represent carbohydrate in actual structural combination. Decreasing values obtained after repeated crystallisation of native proteins should be interpreted with caution, since, it is not improbable that dissociation of the component systems comprising the protein takes place in solution, the carbohydrate-rich fractions being left in the mother-liquors.

My thanks are due to Mr A. T. King for his continued interest in these studies and to Mr A. R. Colwell of the Biochemical Laboratory, Cambridge, for his kindness in performing numerous micro-Kjeldahl determinations.

I also wish to acknowledge the benefit I have derived from correspondence with Prof. P. A. Levene upon the subject of the presence of carbohydrate groups in the serum- and egg-proteins.

REFERENCES.

Bertho, Hölder, Meiser and Hüther (1931). *Liebig's Ann.* **485**, 127.
Bywaters (1909). *Biochem. Z.* **15**, 322.
Dische and Popper (1926). *Biochem. Z.* **175**, 371.
Calvery (1929). *J. Biol. Chem.* **83**, 631.
Gortner (1916). *J. Biol. Chem.* **26**, 177.
Karrer (1930). *Helv. Chim. Acta*, **11**, 1105.
Levene and Mori (1929). *J. Biol. Chem.* **84**, 49.
—— and Rothen (1929). *J. Biol. Chem.* **84**, 63.
Lustig and Haas (1931). *Biochem. Z.* **231**, 487.
Rimington (1929). *Biochem. J.* **23**, 430.
Sørensen (1930). *Compt. Rend. Trav. Lab. Carlsberg*, **18**, No. 5.
Tillmans and Phillipi (1929). *Biochem. Z.* **215**, 36.
Zanetti (1897). *Ann. Chim. Farm.* **12**, 1.
—— (1903). *Gazzetta*, **33**, 160.

CXX. THE RELATION BETWEEN TOTAL SULPHUR AND CYSTINE CONTENT OF CERTAIN SAMPLES OF RABBIT FUR.

By JOHN BARRITT and CLAUDE RIMINGTON.

From the Chemical Laboratories, The Wool Industries Research Association, Torridon, Headingley, Leeds.

(*Received June 22nd, 1931.*)

In a recent study of the effect of diets supplemented with cystine upon the composition of the keratin of rabbit fur, large variations, which it was concluded were seasonal, were found to occur in the total sulphur content of the fur of both control and experimental animals [Barritt, King and Pickard, 1930].

It was considered to be of interest to ascertain whether these increased sulphur values were represented wholly by cystine, since it has been shown [Rimington, 1929, 1, 2] that almost the whole of the sulphur in wool and various animal hairs can be accounted for in this form.

The results indicate that within slight limits, possibly of experimental error, the sulphur in all the samples examined can be accounted for in this form.

Some observations made upon the technique of colorimetric cystine determination emphasise, as detailed below, the instability of this amino-acid and the necessity of observing certain experimental precautions if low values are to be avoided.

Experimental.

The samples of rabbit fur were obtained from a number of Angora rabbits fed on a normal diet to which various additions of cystine were made during an interval of 84 days [Barritt, King and Pickard, 1930]. The material was sampled and purified as previously described [Barritt and King, 1926, 1929].

Approximately 1 g. of fur was hydrolysed with 25 cc. of 20 % hydrochloric acid for 5½ hours. During the initial stages of hydrolysis there was a tendency for violent bumping to occur. Any slight loss of material was not, however, of serious moment since only comparative values were required of total sulphur by the modified Benedict-Denis method, and of cystine by the Folin-Marenzi [1929] method, in the hydrolysate. The values for total sulphur in the samples were obtained by the Carius method [Barritt, King and Pickard, 1930].

Two 5 cc. samples of the hydrolysate were taken for total sulphur determination as described by Rimington [1930, 1]. For determination of cystine a 5 cc. sample of the hydrolysate was diluted to approximately 42 cc. and 5 cc. of 20 % sodium hydroxide were added slowly with constant shaking, this being sufficient to reduce the acidity to approximately $N/10$. The mixture was made up to 50 cc., filtered and 2 cc. portions of this solution taken for the colorimetric procedure using urea to prevent turbidity [Rimington, 1930, 2].

The detailed results of Table I show that the increased total sulphur of the second clip can be accounted for by the higher cystine content of these furs.

Table I.

	Rabbit	Total sulphur (Carius)	Cystine in 5 cc. of hydrolysate (Folin-Marenzi) (mg.)	Cystine in 5 cc. of hydrolysate calculated from determination of total sulphur (Benedict-Denis) (mg.)	Difference (mg.)	Difference (%)
Controls	103 B	5·10	32·5	32·8	−0·3	−1·0
	103 A	5·05	—	36·3	—	—
	243 B	4·64	33·7	35·0	−1·3	−3·7
	243 A	4·98	37·4	36·7	+0·7	+1·9
	302 B	4·12	30·7	30·7	0	0
	302 A	4·79	41·1	41·75	−0·65	−1·6
0·1 g. cystine per day	63 B	4·93	47·1	46·0	+1·1	+2·4
	63 A	5·11	39·7	39·6	+0·1	+0·25
	235 B	4·77	32·3	32·2	+0·1	+0·3
	235 A	4·91	36·4	36·5	−0·1	−0·3
0·2 g. cystine per day	193 B	4·92	38·7	38·9	−0·2	−0·5
	193 A	5·02	41·1	41·8	−0·7	−1·7
	238 B	4·85	35·1	35·4	−0·3	−0·85
	238 A	5·05	37·4	36·4	+1·0	+2·7
	253 B	4·57	36·7	37·2	−0·5	−1·3
	253 A	4·89	37·6	38·1	−0·5	−1·3

Samples marked B were clipped on April 18th, 1929.
Samples marked A were clipped on July 11th, 1929.

The effect of concentration during the neutralisation of the hydrolysate.

It was found advisable to adhere carefully to the above procedure for the colorimetric determinations since, otherwise, anomalous results were obtained. In particular it is essential that the hydrolysate should be largely diluted before addition of the alkali since neutralisation of a concentrated solution, even if carried out with every precaution against rise of temperature, is liable to lead to very appreciable destruction of cystine. In a particular example a 10 cc. sample of the undiluted hydrolysate was treated with 5 cc. of 40 % sodium hydroxide, added drop by drop, the vessel being immersed in cold water and kept continuously agitated. The mixture was filtered and of the almost colourless filtrate 5 cc. were taken for a determination of total sulphur

whilst another 5 cc. were diluted to 50 cc. and used for the colorimetric determination.

The results obtained were as follows:

Total sulphur (Benedict-Denis) equivalent to 19·2 mg. cystine/5 cc. filtrate.
Cystine sulphur (Folin-Marenzi) equivalent to 13·1 mg. cystine/5 cc. filtrate.

Or expressed as cystine in the original hydrolysate:

Total sulphur (Benedict-Denis) equivalent to 28·8 mg. cystine/5 cc. hydrolysate.
Cystine sulphur (Folin-Marenzi) equivalent to 19·6 mg. cystine/5 cc. hydrolysate.

When the hydrolysate was diluted in the normal way, as described above, the following values were obtained which show good agreement between total sulphur and cystine sulphur:

Total sulphur (Benedict-Denis) equivalent to 28·5 mg. cystine/5 cc. hydrolysate.
Cystine sulphur (Folin-Marenzi) equivalent to 28·6 mg. cystine/5 cc. hydrolysate.

It would appear that concentrated sodium hydroxide acting upon the undiluted hydrolysate causes a very appreciable destruction of cystine.

The authors' thanks are tendered to Mr A. T. King, Head of the Chemical Laboratories of the Association, for help and advice rendered during the course of their work.

REFERENCES.

Barritt and King (1926). *J. Text. Inst.* **17**, 394 T.
—— —— (1929). *J. Text. Inst.* **20**, 151 T.
—— —— and Pickard (1930). *Biochem. J.* **24**, 1061.
Folin-Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Rimington (1929, 1). *Biochem. J.* **23**, 41.
—— (1929, 2). *Biochem. J.* **23**, 726.
—— (1930, 1). *J. Soc. Chem. Ind.* **49**, 139 T.
—— (1930, 2). *Biochem. J.* **24**, 1114.

CXXI. NOTE ON THE SULPHUR-FREE NATURE OF MEDULLA IN BLACKFACE WOOL.

By JOHN BARRITT and ALBERT THEODORE KING.

From the Chemical Laboratories, The Wool Industries Research Association, Torridon, Headingley, Leeds.

(*Received June 22nd, 1931.*)

The presence of a medullated core is a well-known characteristic distinguishing the coarser (kempy) types of wool fibre from the fine merino type comprised only of scale and cortex.

Knowledge of the nature and amount of this medullary substance was confined to qualitative microscopic observation prior to the figures derived by Barker and King [1926] from a comparison of observed fibre diameters with those calculated from length, weight and density determinations.

The close agreement of the two methods, when non-medullated fibres are employed, falls off in proportion to the extent of medullation, but this circumstance provides a means of calculating approximately the relative proportions of medulla substance, air space, and non-medullated substance (cortex and scale) contained in a medullated fibre.

Blackface wool fibres were employed, as the remarkable regularity of both medulla diameter and total fibre diameter makes them eminently suitable for the measurements concerned.

From a series of measurements, detailed in the paper referred to, on typical medullated fibres (excluding heavily or only slightly medullated fibres) the following values were calculated:

mean percentage of air space to total fibre volume approx. 55·0 %;

mean percentage of air space to volume of medulla substance approx. 90 %.

Thus the average content of actual medulla substance in these fibres is 5·5 %.

Determinations of sulphur content [Barritt and King, 1926] of the bulk sample of moderately medullated fibres from which the fibres for the above measurements were drawn gave 3·33 %, while that for fine unmedullated fibres from the same portion of the fleece gave 3·82 %. From these values, making the assumption that the sulphur content of the cortex and scale portion is the same for both medullated and unmedullated fibres (*i.e.* 3·82 %) an equation relating the medulla channel and the sulphur content of the actual medulla substance can be derived as follows.

Let X = % volume of medulla channel in total fibre volume,

and Sm = sulphur content of medulla substance,

then $$\frac{X}{10}Sm + (100 - X)\,3{\cdot}82 = \left(100 - X + \frac{X}{10}\right)3{\cdot}33.$$

Hence $$490 = X\,(8{\cdot}23 - Sm).$$

Taking arbitrary values of Sm the following values of X are obtained:

Sm	X
0	59·5
1	67·8
2	78·6
3	94·0

The figure of 55 % obtained by Barker and King for the average air-space in the medullated fibres corresponds to 60·5 % of medulla channel, which is in striking agreement with the calculated figure of 59·5 % assuming zero sulphur content in the medulla substance.

Reviewing the possible sources of error which might combine to make this agreement merely a coincidence, the actual measurements and sulphur determinations are regarded as of sufficient precision to preclude any appreciable error in the figure deduced.

The assumptions necessarily made are, firstly, that the density of the medulla substance is the same as that of the unmedullated fibre. Widely differing types of wool fibre have practically identical density values [King, 1926] and the density of dry gelatin, 1·34 [see Svedberg, 1930] and of protein matters generally is not very different from 1·30 for wool substance, whereas only a wide divergence would seriously affect the result.

Secondly, it has been assumed that if the scales and cortex differ in sulphur content, any variation in the relative proportions of scale and cortex will not seriously affect the sulphur value. This can only be decided when unscaled fibres [King, 1927] can be analysed for sulphur content.

Thirdly, alternatively to the assumption made that the sulphur content of the unmedullated portion is the same as of the non-medullated fibres, a generally lower value in all the components of the medullated fibre would of course equally explain the difference in actual sulphur values. The direct evidence advanced in the foregoing paper however, affords a very strong substantiation of the mathematical deduction that the sulphur content of medulla substance, if not actually *nil*, must be extremely low in comparison with that of scale and cortex.

REFERENCES.

Barker and King (1926). *J. Text. Inst.* **17**, 68 T.
Barritt and King (1926). *J. Text. Inst.* **17**, 394 T.
King (1926). *J. Text. Inst.* **17**, 53 T.
—— (1927). *Biochem. J.* **21**, 434.
Svedberg (1930). *Trans. Far. Soc.* **26**, 691.

CXXII. SULPHUR DISTRIBUTION IN THE COMPONENT STRUCTURES OF WOOL AND PORCUPINE QUILLS.

By JOHAN GODFRIED BEKKER and ALBERT THEODORE KING.

From the Chemical Laboratories, The Wool Industries Research Association, Torridon, Headingley, Leeds.

(*Received June 22nd, 1931.*)

The views originally advanced by one of us [King, 1926] and in more detail by Barritt and King [1929] regarding the biological significance of sulphur variability in wool are, in brief, that, in the first place, "keratinisation" is the result of incorporation of cystine nuclei with the primary plasmic cell-forming material, the extent of this incorporation or "sulphur stimulus" being variable with different types of wool and with different genetic, pathological, and environmental factors, particularly, as regards the last named, diet and seasonal conditions. Further evidence of this variability has been published by Rimington [1929] and Barritt and King [1929] as opposed to the statement of Robertson and Marston [1929] that wool keratin has a constant sulphur content.

Recent work in these laboratories by Bonsma [1931] has established some evidence of correlation of sulphur content with diet and seasonal changes in the case of South African wool, and his discussion of its biological and technical significance is substantially an elaboration of the views already advanced [King, 1929].

Secondly, from indirect evidence, summarised in the first paper above mentioned (details of which are published for the first time in the accompanying note [Barritt and King, 1931]) it was concluded that "the medulla cells...are substantially devoid of sulphur" and that a biological deficiency in "sulphur stimulus" is a factor associated with presence of the medulla, unkeratinised by cystine incorporation, which constitutes the inner core of kempy fibres.

The essential evidence lies in the substantially lower sulphur content of medullated fibres as compared with non-medullated fibres from the same portion of the fleece, and, in view of the fact that recently Rimington [1931] found only comparatively small differences in this respect with certain types of wool, it appeared desirable to examine some hirsute tissue in which the medulla could be separated with some degree of accuracy and independently analysed.

For this purpose porcupine quills, morphologically analogous to wool fibre,

were employed. They were split with a knife, and the medullated core carefully scraped away from the cortex. Complete separation is impracticable on account of the radially disposed lamellae of cortical tissue (see Fig. 1*a*) but, while the medulla remained contaminated with the cortical lamellae, the cortex was obtained substantially free from medulla.

The samples were degreased with boiling benzene, washed in water, and dried to constant weight.

Analysis gave the following results[1].

Ash (whole quill) 0·79 %.

	Whole quill	Cortex	Medulla
Total sulphur	1·31	1·46	1·25
	1·38	1·53	1·23
Mean	1·35	1·50	1·24

The sulphur content for the whole quill is thus 10 % lower than that for the cortex. Typical values for non-kempy and kempy wool fibre (3·82 and 3·33 respectively) show a difference of 12·8 %, a figure of the same order. The actual weight of medulla substance in the wool kemp examined is approximately only 5 % of the total fibre weight. Unfortunately, as mentioned above, the failure to obtain complete separation of the porcupine medulla again precludes direct analytical evidence of its freedom from sulphur, but the microscopic appearance of its very attenuated structure of hollow cells indicates that its proportion by weight of the whole quill must be very small, and the results therefore are not inconsistent with those for medullated wool fibre.

Confirmatory evidence was sought from the action of specific staining agents on sections of both wool and quill.

Sodium plumbate solution (10 minutes treatment in the cold) rather disturbs the structure on account of its alkaline character, but shows clearly the location of the sulphur in the cortex (Fig. 1*b*).

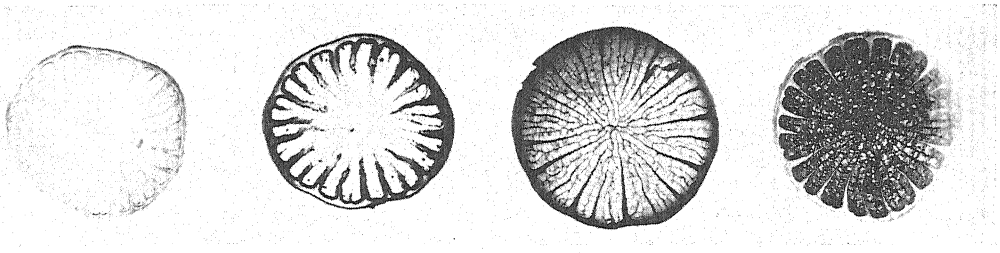
The Sullivan reagent (applied by boiling the quill section in dilute hydrochloric acid for a few minutes to liberate free cystine, and then immersing in sodium naphthaquinone-4-sulphonate) shows a slight diffusion of stain, which is however again essentially confined to the cortex (Fig. 1*c*).

The Pauly reagent (diazotised sodium sulphanilate) not only distinguishes clearly the structural form of the medulla from the lamellae of the cortex, but indicates chemical differences as well.

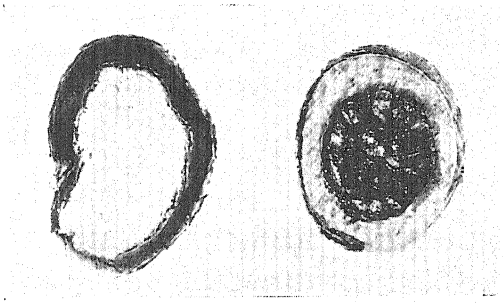
This reagent is well known to give similar brown-red colorations with a number of the naturally-occurring amino-acids, but according to Totani [1915] when the stain originally developed is treated with zinc and hydrochloric acid, with subsequent addition of ammonia until alkaline, only the tyrosine and histidine stains remain, the former rose-red, the latter golden yellow. Moreover on oxidation of these secondary colorations with hydrogen peroxide, the tyrosine colour disappears leaving only a lemon-yellow histidine coloration,

[1] We are indebted to Dr C. Rimington for these analyses.

which is stable to this oxidising agent. Convenient practical details for these tests are given by Burgess and Rimington [1929].



Unstained *a* | Lead acetate *b* | Sullivan reagent (cystine) *c* | Pauly reagent (tyrosine) *d*



Wool fibre (Kemp)

Lead acetate *e* | Pauly reagent *f*

Fig. 1.

With sections of non-medullated wool fibres these colour changes are readily followed, the final lemon-yellow histidine stain being distributed throughout the cortex. Sections of medullated wool fibre however behave anomalously, in that the pronounced red-brown stain of the medulla is resistant both to the reducing and oxidising actions. Thus elimination of the tyrosine stain leaves a more brownish stain, equally persistent with the histidine stain remaining in the cortex, and masking any residual histidine stain which may also be given by the medulla (Fig. 1*f*).

Porcupine quills show similar behaviour, though the residual histidine stain of the cortex is very faint compared with that of wool fibre (Fig. 1*d*).

The photographs of course give a very inadequate impression of the striking colour differences.

Thus the chemical distinctions in the various components of wool fibre may be summarised:

Scales. Cystine, positive; tyrosine (and ? histidine), negative.
Cortex. Cystine, positive; tyrosine and histidine, positive.

Medulla. Cystine, negative; tyrosine (and ? histidine), positive; also specific reaction to Pauly reagent not shown by scale or cortex, indicating some constituent, as yet unidentified, peculiar to the medulla.

In contrast, picric acid, regarded as a specific keratin stain, produces no distinguishing effects, but a general stain for scale, cortex and medulla alike.

Thus the differential staining effects with both wool and quill sections confirm the view previously advanced that medulla cells are substantially devoid of sulphur, and that keratinisation, so far as scleroproteins such as wool keratin are concerned, is characteristically the result of incorporation of the cystine nucleus.

We therefore prefer to regard the term "keratinisation" as synonymous with cystinisation in contrast to general hardening or cornifying effects occurring in protein structures.

If we accept the histological view that scale, cortex, and medulla are all developed from the same primary plasmic material of the basal layer of the epidermis, the cornification process must be different in each case. That of the scale cells essentially involves true keratinisation or cystinisation, apparently without incorporation of the tyrosine nucleus; that of the cortex involves both cystine and tyrosine incorporation, while the hardening of the medulla cells, mainly a drying up of the semi-fluid contents to form hollow cells, involves in addition a certain degree of cornification associated with some keratinising agent (using the term in the general sense) other than cystine, the nature of which must await further chemico-histological study of the follicle and skin tissues.

In conclusion the authors express their thanks to Mr C. G. Winson, Miss Walker and Miss Hind for their valuable assistance with the photographic reproductions.

REFERENCES.

Barritt and King (1929). *J. Text. Inst.* **20**, 151 T.
—— —— (1931). *Biochem. J.* **25**, 1075.
Bonsma (1931). *J. Text. Inst.* **22**, 305 T.
Burgess and Rimington (1929). *J. Roy. Microscopical Soc.* **49**, 341.
King (1926). *J. Text. Inst.* **17**, 394 T.
—— (1929). *Text. Merc. Oct.* **23**, 17.
Rimington (1929). *Biochem. J.* **23**, 726.
—— (1931). *Biochem. J.* **25**, 71.
Robertson and Marston (1929). Council of Scientific and Industrial Research of Australia, Bulletins 38 and 39.
Totani (1915). *Biochem. J.* **9**, 385.

CXXIII. VITAMIN C IN THE ORANGE AND THE GRAPE FRUIT.

By MARY FORREST BRACEWELL
and SYLVESTER SOLOMON ZILVA[1].

From the Division of Nutrition, Lister Institute, London.

(*Received June 22nd, 1931.*)

Introduction.

The experiments described in this report are a part of a general investigation concerned with the bearing of the antiscorbutic factor on the biological activity of the plant. They have been carried out in a similar way to those made in the study of this vitamin in the apple [Bracewell, Hoyle and Zilva, 1930; Bracewell, Kidd, West and Zilva, 1931; Bracewell, Wallace and Zilva, 1931], with the aim of correlating the antiscorbutic activity of the fruit with its environment.

In this communication the influence of the season on the potency of the orange and of season and variety on that of the grape fruit has been investigated. It is evident that carefully controlled material which could be conveniently transported to meet the requirements of the enquiry was necessary and with this end in view Palestine was chosen as the source of the orange and British Honduras and South Africa as those of the grape fruit.

Concordant results were obtained during two consecutive seasons and indications are available that the time of picking has no influence on the activity of the juice of the orange or of the grape fruit, and in the case of the latter no very significant difference was observed in the potency of the juice of the several varieties investigated. There is some evidence, however, that those fruits, which yield a diminished amount of juice with the progress of the season and on storage, also yield a correspondingly smaller amount of vitamin since there is no change in the minimum protective dose of the juice. This would suggest that the antiscorbutic factor or one or more of the substances which contribute to its stability [Zilva, 1927, 1928, 1929, 1930] are utilised in the process of ageing of the fruit.

Technique.

The method of assay of vitamin C was the same as that employed in the previous investigation [Bracewell, Hoyle and Zilva, 1930]. The test was continued in most cases for 90 days. A prolonged test was important in these experiments since any serious deterioration in the antiscorbutic activity of a

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

batch of fruit during a short period of storage would in all probability have been reflected in the weight curves.

The antiscorbutic activity of the Jaffa (Shamuti) orange.

Oranges are exported from Palestine from November to April inclusive; the bulk of the fruit is normally shipped in December, January, February and March. It was therefore desirable to examine samples collected at the beginning, middle and end of the season. This was done in the first experiment in 1928/29. During the following season material from the beginning and from the end of the season only was tested for confirmatory purposes.

Nine adjacent trees, planted at 5·5 metres distance and bearing 27 cases of fruit, in a grove situated 3 miles north of Jaffa, were set aside for the experiment. Fruit was picked from all the nine trees at the different periods of the season. The trees, about 25 years old, were healthy and were budded on bitter orange stock (*Citrus bigaradia*) in their second year. They were growing on fairly heavy loamy soil. The manurial treatment was as follows.

To each tree was added:

100 kg. old horse manure in October, 1927.
1·125 ,, sulphate of ammonia in March, 1928.
,, ,, ,, ,, June, 1928.
,, ,, ,, ,, August, 1928.
100 ,, old horse manure in October, 1928.

The same group of trees was used for the 1928/29 and 1929/30 experiments. Table I gives the dates of picking *etc.* In the 1929/30 season the oranges were

Table I.

Batch	Picked	Beginning of biological test
V 1, 1928	Early November	11. xii. 28
V 4, 1929	Early January	5. ii. 29
V 9, 1929	Early March	15. iv. 29
V 1, 1929	Early December	2. i. 30
V 9, 1930	Early March	2. iv. 30

exceptionally late in maturing and consequently the first batch was picked about 3 weeks later than the corresponding batch of the previous year.

The tests for vitamin C were begun immediately on the arrival of the fruit. The oranges were stored at a temperature of about 15° during the three months' duration of the test. The juice was expressed daily before dosing and the quantity yielded by each fruit noted.

The chemical constitution of orange juice changes when the fruit is held on the tree and when kept in storage. During the process of ripening the soluble solids content of the juice increases, especially during the last 2 or 3 weeks [Copeman, 1931]. There are also a gradual increase of total sugar, sucrose and reducing sugars increasing at about the same rate, and a decrease of acidity as the oranges mature [Collinson, 1913].

Circumstances did not permit in this investigation of many chemical analyses being made, but the changes observed were broadly in agreement with those previously recorded by workers who had studied this aspect of the subject in greater detail.

Table II gives a 10-day average of the yield of juice per orange of the

Table II. *Daily average yield of juice per orange. The duration of the test is divided into 10-day periods.*

Period	V 1, 1928 cc.	V 4, 1929 cc.	V 9, 1929 cc.	V 1, 1929 cc.	V 9, 1930. A cc.	V 9, 1930. B cc.
1	67·60	65·70	42·46*	66·66	53·33	63·33
2	65·40	57·24*	50·75	69·00	48·50	65·50
3	63·70	68·29*	43·78	67·25	50·00	70·00
4	79·10*	60·43	41·50	61·30*	45·00*	60·00*
5	58·88*	54·62	41·11	65·33	53·01	58·33*
6	63·55	62·17	35·50	58·00	35·00	51·66
7	43·14	61·50	32·11	50·00	—	51·33
8	43·90	58·87	20·52	45·10	—	48·30

* Denotes period during which the juice was analysed. Results are given in Table III.

various batches as the tests proceeded. It is seen that the yield decreases as the test of each batch proceeds. It decreases further as the season advances. The last batch (V 9) of the 1929/30 season arrived in two different containers, A and B, and the oranges from each box were removed on alternate days for the tests so as to obtain an average result. It will be seen, however, that there was a marked difference in yield of juice by the fruit of the two consignments,

Table III.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. Citric acid/ 100 cc.	Maturity ratio	p_H of juice
V 1, 1928	12. i. 29	9·49	1·35	7·1	3·06
Early November		8·72	1·29	6·8	3·11
		8·11	0·68	12·0	3·62
	25. i. 29	9·39	1·32	7·2	3·13
		9·43	1·27	7·4	3·23
V 4, 1929	19. ii. 29	10·93	0·94	11·6	3·29
Early January		12·00	0·98	12·3	3·35
		9·86	1·37	7·2	3·23
	6. iii. 29	9·32	0·94	10·0	3·33
		9·83	1·10	8·9	3·23
V 9, 1929	15. iv. 29	12·50	0·73	17·0	3·75
Early March		11·89	0·77	15·4	3·64
		12·81	0·46	27·7	3·95
	18. iv. 29	10·40	0·62	16·9	3·72
		11·55	0·33	35·4	4·20
V 1, 1929	12. ii. 30	10·42	0·79	13·15	3·02
Early December		8·70	0·93	9·34	2·87
		9·02	0·79	11·58	2·99
V 9, A 1930	13. v. 30	10·57	1·17	9·06	3·45
		11·24	0·85	13·32	3·60
B 1930	9. v. 30	10·21	0·56	15·66	3·90
Early March		11·89	0·79	15·02	3·72
	18. v. 30	10·61	0·95	11·23	3·48
		10·76	1·02	10·50	3·72

although they were picked at the same time and in the same grove. The average yield of the two is lower than that from the first batch, V 1, of this season.

Table III sets out the analytical data of the various batches. Unfortunately, as will be seen from Table II, the analyses could not be carried out in corresponding periods of the testing time. Nevertheless, they give a fair survey of the changes that took place in the composition of the juice with the progress of the season. It will be seen that in the 1928/29 season there was an increase in the soluble solids as the oranges remained longer on the tree. The acid content and the hydrogen ion concentration decreased. The maturity ratio (ratio of soluble solids to acidity calculated as citric acid) rose in consequence as the season advanced. The figures for the season 1929/30 did not show quite the same trend, but this is possibly due to the abnormality of the season.

The vitamin C assays (Table IV) do not reveal any marked difference in

Table IV. *Antiscorbutic potency of juice from Jaffa oranges.*

Dose cc.	V 1, 1928 Picked early November	V 4, 1929 Picked early January	V 9, 1929 Picked early March	V 1, 1929 Picked early December	V 9, 1930 Picked early March
½	No protection	No protection	?	+	±
1	+ + +	+ + +	+ + + ±	+ + + ±	+ + + ±
1½	Not tested	+ + + ±	+ + + +	+ + + +	+ + + +
2	+ + + +	+ + + +	+ + + +	Not tested	Not tested
3	+ + + +	Not tested	Not tested	+ + + +	+ + + +

potency within the limits of the gradation of the tested doses. There was possibly a slightly higher potency in the juices of V 9, 1929 and V 1 and V 9, 1930. This disparity is, however, of such a low order that it cannot be considered significant. The appearance of the weight curves was normal and the *post mortem* findings were consistent, which suggests that a significant loss in the antiscorbutic potency was not incurred during at least the first two months of the test.

The antiscorbutic activity of grape fruit from British Honduras.

The export season in British Honduras lasts from October to the beginning of April, and samples were examined which were collected at the beginning, the middle and the end of the season during the first year and at the beginning and at the end of the season during the second year. Two varieties, namely Duncan and Marsh, were investigated. Owing to a misunderstanding the two varieties were mixed in the first season's tests. The following year however, each variety was tested separately in the autumn in order to ascertain whether they differed in potency.

The fruit was collected from two healthy trees from a grove in British Honduras situated 14 miles from the sea coast on the lower slope of a hill at an altitude of about 100 ft. above sea level. The trees were imported from the United States and were planted in the present grove in 1914. The age of

the trees and nature of the stock is not known. The tree from which the Duncan variety was picked is grown in 18 in. of reddish-brown, alluvial silt grading to a dry, dense bright red micaceous clay subsoil, p_H 6·9. The Marsh variety is grown on 14 in. of reddish brown, alluvial silt grading to a reddish yellow, gritty and micaceous dry clay subsoil, p_H 6·9. Grass grows throughout the grove which is grazed by mules. Grazing has caused the surface to become somewhat packed. The trees were sprayed with Bordeaux sulpho-citrol four times a year. 15 lb. per tree of a manurial mixture containing 10 % of phosphoric acid, 6 % nitrogen and 7 % of soluble potash was forked in during March and April.

The vitamin tests were begun immediately on the arrival of the fruit, which was stored at about 15°. The juice was expressed daily, but owing to the size of the fruit only a part of it was used. The remainder was kept in cold store at 0° until the fruit was finished, usually 3 days. Table V gives dates of picking and the beginning of the tests.

Table V.

Batch	Picked	Beginning of biological test
G 1, 1928. Marsh and Duncan	Early December	14. i. 29
G 3, 1929. Marsh and Duncan	Early January	20. iii. 29
G 4, 1929. Marsh and Duncan	Early March	10. iv. 29
G 1, 1929. Marsh	Early October	12. xi. 29
G 1, 1929. Duncan	Early October	13. xi. 29
G 4, 1930. Marsh	Early April	13. v. 30

The changes which take place in the chemical constitution of the grape fruit juice are briefly the following. Collinson [1913] found a decrease in acidity and an increase in the sugar content as the season advanced. After the fruit has matured the sucrose is gradually hydrolysed to reducing sugar. Zoller [1918] observed a decrease of acid content during storage and an increase of sugar after the removal of the fruit from the tree. According to Hawkins and Magness [1920] the acid content decreases and there is an apparent increase in sugar during cold storage. The dry matter increases during storage. There is a shrinkage from 5–8 % in cold storage to 25 % in warm ventilated storage. Hawkins [1921] found further that in warm storage there is a marked increase in the percentage of the acid calculated on the wet weight of the pulp, which is entirely due to loss of water. There is also possibly a slight decrease in the sugar content. In cold storage the acidity decreases markedly after 4 months whilst there is little change in the sugar content.

Table VI presents the analytical data obtained from the fruit used in this investigation. No very marked difference between the various periods can be discerned from these few data. The figures, however, suggest that the soluble solids and the acid content of the Duncan variety of 1929/30 season were lower than those of the Marsh variety of the same season.

The antiscorbutic potency of the grape fruit is summarised in Table VII. The general evidence points to the fact that this fruit, too, does not vary

Table VI.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. citric acid/ 100 cc.	p_H of juice
G 1, 1928	28. ii. 29	9·76	1·03	3·16
Marsh and Duncan		10·43	0·58	3·65
Early December	4. iii. 29	11·45	0·74	3·23
		10·83	0·76	3·30
G 3, 1929	7. iii. 29	10·53	0·88	3·32
Marsh and Duncan		12·97	1·18	3·18
Early January	23. iii. 29	10·12	0·75	3·43
		10·27	0·66	3·51
G 4, 1929	10. iv. 29	9·89	0·87	3·35
Marsh and Duncan		9·97	0·62	3·62
Early March	16. iv. 29	11·34	0·73	3·48
		11·61	0·76	3·44
G 1, 1929	8. i. 30	10·24	1·17	3·29
Marsh		10·56	1·10	3·20
Early October				
G 1, 1929	8. i. 30	8·90	0·89	3·16
Duncan		8·81	0·69	3·31
Early October				
G 4, 1930	16. vi. 30	10·08	0·95	3·25
Marsh		10·45	1·00	3·38
Early April		10·26	1·09	3·29

Table VII. *Antiscorbutic potency of juice from grape fruit. British Honduras.*

Dose cc.	G 1, 1928 Marsh and Duncan Picked early December	G 3, 1929 Marsh and Duncan Picked early January	G 4, 1929 Marsh and Duncan Picked early March	G 1, 1929 Marsh Picked early October	G 1, 1929 Duncan Picked early October	G 4, 1930 Marsh Picked early April
½	+ ±	?	?	+ +	+ + +	+ ±
1	+ + + + ?	+ + +	+ + +	+ + + +	+ + + +	+ + + + ?
1½	Not tested	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
2	+ + + +	+ + + +	+ + + +	Not tested	Not tested	Not tested
3	+ + + +	Not tested	Not tested	Not tested	Not tested	+ + + +

within the limits of the dosage used in these tests, with the time of season. Nor do the weight curves reveal a very marked loss in activity during the first 2 months of the test period. The results call, however, for some comment. The mixed juices of the Marsh and Duncan of December 1928 showed a somewhat higher vitamin content than did those of January and of March 1929. The difference in activity is of an order which falls outside the limits of the experimental error. It is difficult, however, to accept the view that this falling off in activity was due to the longer sojourn of the fruit on the tree, since no significant difference was observed between the activity of the Marsh variety picked in October and that of this fruit picked in April of the following season.

A somewhat marked falling off in weight of the guinea-pigs on the 1 cc. dose of the Marsh picked in April 1930 was observed towards the end of the test. This could hardly be due to the deterioration of the activity of the fruit during the test period, since the manifestation of scorbutic symptoms should

have preceded the decline in weight if it were due to scurvy. This did not occur except in one animal. Nor did the *post mortem* examination of the animals suggest that the loss in weight was due to scurvy.

It must be noted that the Duncan variety, in spite of lower total solids, showed a somewhat higher vitamin content than the Marsh variety.

THE ANTISCORBUTIC ACTIVITY OF GRAPE FRUIT FROM SOUTH AFRICA.

These experiments were instituted in order to ascertain whether there is a difference in the antiscorbutic potency of the same variety (Marsh) emanating from a different environment. An additional variety (Florida) was also assayed.

The South African grape fruit season lasts from the middle of May to the middle of August. The two varieties were received early in September.

Table VIII. *Antiscorbutic potency of juice from grape fruit. South Africa.*

Dose (cc.)	1930. Marsh No protection	1930. Florida
½		±
1	+ + + ±	+ + + ±
1½	+ + + +	+ + + +
3	+ + + +	+ + + +

The Marsh variety was grown at Simondium, Western Cape, on Karroo silt. The trees were 12 years old, healthy and budded on Rough Cape Lemons. They were planted in the grove in 1918. They were previously bad with scale, but are fairly clear now and were not sprayed. The soil was ploughed annually and has been irrigated six times annually since March 1928. Previous to this, owing to drought, irrigation was erratic, the trees going sometimes for as long as six months without water. The irrigation was followed by cultivation. They have been fertilised each autumn with kraal manure since 1926.

The Florida variety was grown in the Eastern Cape Province on Karroo silt. The trees were 8 years old, healthy and budded on Rough Cape Lemons. They were planted in 1922 and were not sprayed. The method of irrigation, cultivation and manuring was the same as for the Marsh variety.

As will be seen from Table IX, these batches of grape fruit had a higher

Table IX.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. citric acid/100 cc.	p_H of juice
1930. Marsh	2. x. 30	10·31	1·44	2·71
	14. x. 30	9·67	1·46	2·80
	17. x. 30	10·18	1·78	2·83
1930. Florida	2. x. 30	8·69	1·69	2·83
	14. x. 30	8·51	1·78	2·87
	17. x. 30	8·26	1·76	2·71

acid content and lower p_H value than the British Honduras varieties. The Florida variety showed a total solids content similar to that of the Duncan variety from British Honduras. The vitamin content (Table VIII) was of the

same order as that of the corresponding British Honduras Marsh variety. It is true that the South African Marsh variety shows slightly lower activity than the one of British Honduras origin, but the difference is too small to be considered significant under the circumstances.

Conclusions.

These systematic observations on the constant antiscorbutic activity of the orange and grape fruit during the entire season and on the stability of the vitamin in the intact fruit are consistent with the scattered observations made by various workers in this connection. The vitamin C activity of the orange, lemon and grape fruit has very seldom been found to vary to any great extent and evidence is also available concerning its stability. Thus Davey [1921] observed that 5 months of cold storage did not appreciably impair the vitamin C content of the orange. The activity of the orange, as we have seen from the foregoing experiments, like that of the grape fruit, does not seem to fluctuate markedly with the variety. The minimum dose necessary to protect the guinea pig from scurvy, as found in this investigation, is about the same as that recorded by Delf [1921] and Davey [1921] for Denia and Navel oranges. Taking all the available information into consideration one may safely assume that oranges and grape fruit bought casually in the open market under normal conditions ought to possess the full antiscorbutic value characteristic of these fruits.

A point of academic interest which requires further investigation is whether the total amount of vitamin of the Citrus fruits diminishes as they age on the trees or in storage. The yield of juice of the Jaffa orange diminished with the progress of the season when the fruit was left on the tree and during the 3 months' storage in the course of carrying out the test. The antiscorbutic potency of the juice on the other hand did not show an increase. The difference in the yield of the orange juice between the first and last pickings of the 1928/29 season ranged from 25 to 53 % for the corresponding periods, the average being 35 % (Table II). A similar increase in the potency in the juice should have been reflected in the test. A closer spacing of the doses would most probably establish definitely whether or not such an increase takes place.

The small disparity in the vitamin C content of the orange, grape fruit, lemon and sweet lime (*Citrus limetta*) as contrasted with the West Indies lime (*Citrus medica* var. *acida*), which possesses a definitely lower antiscorbutic activity than the aforementioned Citrus fruits, presents an analogy to the variation in the antiscorbutic activity amongst the apple varieties. In the apples, the antiscorbutic activity of which is on a lower plane than that of the Citrus fruits, the Bramley's Seedling, and, according to evidence which is now forthcoming, also some other varieties are markedly more potent than another category of apples such as Cox's Orange Pippin, Worcester Pearmain *etc.*, which differ amongst themselves comparatively little [Bracewell, Hoyle

and Zilva, 1930]. This sharp division in antiscorbutic activity amongst closely related fruits is rather striking and calls for further attention.

Summary.

1. The antiscorbutic activity has been determined of (*a*) the Jaffa orange picked from the same trees at different times of the season during two consecutive years, (*b*) of the Marsh and Duncan varieties (British Honduras) of grape fruit also picked at different times of the season during two consecutive years and (*c*) the Marsh and Florida varieties (South Africa) of grape fruit picked at the end of the season.

2. Within the limits of the doses tested and of the accuracy of the method employed, the vitamin content of the juice is the same whether the orange or grape fruit is picked at the beginning or at the end of the season.

3. There is no marked loss in the vitamin content when the fruit is stored under ordinary conditions at about 15° for about 2 months.

4. The conditions of cultivation, origin of stock, age of tree, soil *etc.* do not affect the vitamin C content of the fruit.

5. The Marsh variety of grape fruit whether of British Honduras or of South African origin, is of approximately the same potency.

6. The Duncan variety of grape fruit (one test only) was found to be slightly more active than the other two varieties of grape fruit tested.

7. The vitamin C content of the grape fruit was rather higher than that of the Jaffa orange.

8. There is no correlation between the soluble solids, acid content, p_H of the juice and its antiscorbutic potency.

We should like to take this opportunity of acknowledging our indebtedness to Mr A. C. Shill, of the Empire Marketing Board, and Mr S. T. Tolkowsky, for collecting and supplying the details for the Jaffa oranges, to Mr H. P. Smart, Agricultural Officer at British Honduras, and to both Mr J. Dimond, Trade Commissioner for South Africa, and Mr Harlow, of the South African Co-operative Citrus Exchange, Ltd., for similar help with the grape fruit. Our thanks are also due to Mrs M. A. Boas-Fixsen for helping us with some of the biological tests.

REFERENCES.

Bracewell, Hoyle and Zilva (1930). Special Report No. 146, Medical Research Council.
—— Kidd, West and Zilva (1931). *Biochem. J.* **25**, 138.
—— Wallace and Zilva (1931). *Biochem. J.* **25**, 144.
Collinson (1913). *Flor. Agric. Exp. Sta. Bull.* **115**.
Copeman (1931). *Trans. Roy. Soc. South Africa*, **19**, 107.
Davey (1921). *Biochem. J.* **15**, 83.
Delf (1921). Pub. South African Inst. for Med. Res. No. 14.
Hawkins (1921). *J. Agric. Res.* **22**, No. 5, October.
—— and Magness (1920). *J. Agric. Res.* **20**, 357.
Zilva (1927). *Biochem. J.* **21**, 689.
—— (1928). *Biochem. J.* **22**, 779.
—— (1929). *Biochem. J.* **23**, 1199.
—— (1930). *Biochem. J.* **24**, 1687.
Zoller (1918). *J. Ind. Eng. Chem.* **10**, 346 (quoted by Hawkins and Magness).

CXXIV. THE CHLORIDES AND INORGANIC CONSTITUENTS OF THE SERUM AND CEREBROSPINAL FLUID IN NEPHRITIS AND ACIDOSIS.

By GEOFFRY CHALLEN LINDER and EDWARD ARNOLD CARMICHAEL.

From the Medical Unit, St Bartholomew's Hospital, London, and The Department of Pathology, University of Capetown.

(*Received June 11th, 1931.*)

In view of the constant relationship of the chlorides of the cerebrospinal fluid to the chlorides of the blood-serum in normal conditions [Hamilton, 1925] and in meningitis [Linder and Carmichael, 1928], it appeared to us of interest to investigate the conditions which obtain in nephritis and in ammonium chloride acidosis. The few observations which we have found in the papers of Hamilton and of Pincus and Kramer [1923] have not revealed any striking changes.

It has been reported [Greenfield and Carmichael, 1925] that in uraemia the cerebrospinal fluid chloride may rise from the normal level of 720–745 mg./100 cc. to 900 or even 1000 mg./100 cc. We have found no record of serum analyses in these cases, but, if we assume the normal relationship to hold, the serum-chloride corresponding to these figures would be 745 and 830 mg. per 100 cc. of serum (127 and 142 *mM*). That this is the case with any but the lesser increases seems highly improbable, for Bulger, Peters, Eisenman and Lee [1926] in 77 cases of nephritis found 5 with a serum-chloride exceeding 100 *mM* and none exceeding 128 *mM*, and Atchley and Benedict [1930] found that a decrease in serum-chlorides and total serum-electrolytes was the rule in uraemic acidosis. No patient presenting such gross increase in cerebrospinal fluid chloride has been available for us to study, but the suggestion that the usual relationship of cerebrospinal to serum-chlorides is abrogated was supported by one of our observations.

Experimental.

Cerebrospinal fluid and arterial blood-serum were obtained and investigated by the methods described in our earlier paper [1928] with the exceptions that the inorganic phosphorus was determined by the method of Fiske and Subbarow [1925], and that the water content of the serum was calculated from a serum-protein determination by the formula, water = (990 − 0·8 × protein) g. per litre [Van Slyke, Wu and McLean, 1923]. As before the concentration of the inorganic constituents was recorded in *mM* concentration in serum-water and in cerebrospinal fluid.

Table I.

Case no.	Diagnosis	Blood urea (mg./100 cc.)	Blood pressure (mm. Hg)	Chloride: Serum-water (mM)	Chloride: CSF (mM)	Cl_s/Cl_{csf}	Bicarbonate: Serum-water (mM)	Bicarbonate: CSF (mM)	$BHCO_{3sw}/BHCO_{3csf}$	Total base: Serum-water (mM)	Total base: CSF (mM)	B_{csf}/B_{sw}	Inorganic phosphorus: Serum-water (mM)	Inorganic phosphorus: CSF (mM)	Protein: Serum (g./100 cc.)	Protein: CSF (g./100 cc.)	Other acids: Serum-water (mM)	Other acids: CSF (mM)	p_H: Serum	p_H: CSF	Remarks
I	Hydraemic nephritis	116	125/60	119	129	0·92	18·8	20·7	0·91	171	162	0·95	2·0	1·1	3·5		22	11	7·38	7·41	Urea therapy
II	Azotaemic nephritis	95	265/160	98	116	0·85	33·5	28·65	1·17	170	161	0·95	1·8	0·7	8·1	0·02	17	15	7·52	7·37	Alkali therapy
III	Azotaemic nephritis, uraemia	200	180/120	118	130	0·91	12·5	17·8	0·70	164	152	0·93	2·1	0·3	7·9	0·03	13	4	7·23		
IV	Azotaemic nephritis, uraemia	380	240/120	98	127	0·77	13·0	13·2	0·98	164	(133)		4·7	5	7·0	0·09	26		7·26		
	Mean normal			111	124	0·89	25	21	1·19	164	156	0·95	1·2	0·6	—	—	—	—	—	—	Hamilton's series

Table II.

Exp.	Chloride: Serum-water (mM)	Chloride: CSF (mM)	Cl_{sw}/Cl_{csf}	Bicarbonate: Serum-water (mM)	Bicarbonate: CSF (mM)	$BHCO_{3sw}/BHCO_{3csf}$	Total base: Serum-water (mM)	Total base: CSF (mM)	B_{csf}/B_{sw}	Inorganic phosphorus: Serum-water (mM)	Inorganic phosphorus: CSF (mM)	Protein: Serum (g./100 cc.)	Protein: CSF (g./100 cc.)	Other acids: Serum-water (mM)	Other acids: CSF (mM)	p_H: Serum	p_H: CSF	Remarks
I a	105	122	0·86	32	27·6	1·16	157	154	0·97	1·5	0·55	6	0·09	3	3	7·45	7·44	Before NH_4Cl
b	117	127	0·92	20·1	23·4	0·86	154	158	1·03	1·55	0·6			− 1	7	7·35	7·38	After 2 days on 12 g./day
c	111	127	0·88	20·15	22·2	0·91	155	154	0·99	1·18	0·47	6·5		1	4·5	7·41	7·40	After 4 days on 12 g./day
II a	107	120	0·89	30·5	26·7	1·14	160	150	0·92	1·18	0·48	7	0·05	2	2	7·52		Before NH_4Cl
b	114	123·5	0·92	22·7	22·8	1·00	161	151	0·94	0·95	0 47	9	0·04	3	5	7·43		After 2 days on 12 g./day

Nephritis.

Table I presents the results obtained in four cases of nephritis. The mean of Hamilton's series is given as a guide to the normal.

Chloride. In one patient, Case II, the cerebrospinal fluid chloride was less than normal. The serum-chloride was also low and so the ratio of Cl_{sw} to Cl_{csf} was the same as in the normal series. The bicarbonate was rather high, and both of these abnormalities were attributed to the fact that until the preceding day the patient had been taking large doses of alkali.

In the remaining patients the cerebrospinal fluid chloride was just above the normal range. In two, Cases I and III, there was a proportionate increase in the serum-water chloride, but in the third, Case IV, the cerebrospinal fluid chloride was high whereas the serum-water chloride was low, and the ratio Cl_{sw}/Cl_{csf} fell to the very unusual figure of 0·77. Such a low ratio appears to be exceptional. Among Hamilton's observations there were two as low as 0·83, one from a case of cerebral tumour and the other from a case of uraemia. In the latter the cerebrospinal fluid chloride was high, 142 *mM* (830 mg./100 cc.); the serum-bicarbonate was 15 *mM*, so there was a moderately severe acidosis. In our own case there was advanced acidosis, the serum-bicarbonate being 12·1 *mM*, the p_H 7·26 and the inorganic phosphorus 4·4 *mM* (13·6 mg./100 cc.).

Bicarbonate. The serum-bicarbonate was very low in Cases III and IV, and was below normal in Case I; in the remaining patient it was high, probably as a result of alkali therapy.

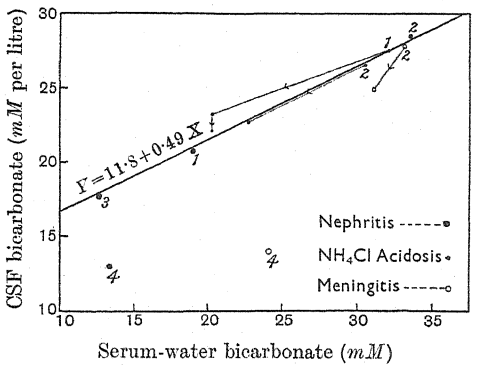


Chart showing relation of serum-water bicarbonate to cerebrospinal fluid bicarbonate. The numbers correspond to the case numbers. The points joined by thin lines are from the same case and the arrows indicate the order in which the points are to be read. Two meningitis cases with very high bicarbonate are omitted.

Hamilton found that the serum-water bicarbonate (X) showed a relationship to the cerebrospinal fluid bicarbonate (Y), which was expressed best for his series by the equation $Y = 9{\cdot}7 + 0{\cdot}46\,X$, X and Y being measured in *mM*. We have plotted the points for our observations in meningitis [1928], nephritis and ammonium chloride acidosis, and found that they tended to lie along a line parallel to but a little higher than Hamilton's; our line corresponded

to the equation $Y = 11{\cdot}8 + 0{\cdot}49\,X$. Three points out of 14 lay far away from this line; two were from meningitic patients, one having a very high and the other a very low serum-bicarbonate, and the third was Case IV of this paper, who had severe acidosis and the abnormal chloride ratio commented upon above.

It is noteworthy that in Case III a still greater degree of acidosis was present, but both the cerebrospinal fluid chloride and bicarbonate were at the levels expected from the normal chloride ratio and the bicarbonate equation.

Total Base. There was no abnormality of total base in these cases, but in Case IV the determination in the cerebrospinal fluid was obviously erroneous and unfortunately could not be repeated.

Ammonium chloride acidosis.

Table II gives the results of producing an acidosis with ammonium chloride in two patients with quiescent tabes. Twelve grams of ammonium chloride were given daily. Cerebrospinal fluid and arterial blood were taken before and after two and four days' treatment. The results were a reduction of serum-bicarbonate by a third, a moderate fall in p_H, and a rise in serum-chloride and serum-protein. The cerebrospinal fluid chloride followed the serum-chloride with possibly a slight delay; the ratio Cl_{sw}/Cl_{csf} became slighly greater, but the normal balance was maintained. The bicarbonate figures followed the line discussed above very closely. The total base ratio was not disturbed.

Summary and conclusions.

It is apparent from these figures that in moderate disturbances of acid-base balance due to nephritis or to ammonium chloride therapy the normal relationships of the cerebrospinal fluid and arterial blood-serum remain intact.

In severe nephritis with great reduction in serum-bicarbonate and p_H and gross nitrogen retention a breakdown of these relationships may sometimes occur as in Case IV and in Hamilton's case McG-n, in which the cerebrospinal fluid chloride was considerably higher than was compatible with the serum-chloride. In Case IV, too, the cerebrospinal fluid bicarbonate was considerably less than was to be expected from the serum-bicarbonate, and the inorganic phosphorus in the cerebrospinal fluid was vastly increased and practically equal to that in the serum. We are of the opinion that these observations give the key to the situation in those cases of uraemia in which very high cerebrospinal fluid chlorides have been reported and explain why such high cerebrospinal fluid chlorides are of evil prognosis.

We wish to express our gratitude to Prof. F. R. Fraser, Prof. A. W. Falconer, and Dr George Graham for permitting us to make observations on their patients, and to the Medical Research Council for personal grants received during part of the time this work was in progress.

REFERENCES.

Atchley and Benedict (1930). *J. Clin. Invest.* **9**, 265.
Bulger, Peters, Eisenman and Lee (1926). *J. Clin. Invest.* **2**, 213.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Greenfield and Carmichael (1925). The cerebro-spinal fluid in clinical diagnosis, p. 75 (Macmillan, London).
Hamilton (1925). *J. Biol. Chem.* **65**, 101.
Linder and Carmichael (1928). *Biochem. J.* **22**, 46.
Pincus and Kramer (1923). *J. Biol. Chem.* **57**, 463.
Van Slyke, Wu and McLean (1923). *J. Biol. Chem.* **56**, 765.

CXXV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

VIII. THE METABOLISM OF GLUTATHIONE COMPARED WITH THAT OF OTHER CYSTINE DERIVATIVES.

By THOMAS SHIRLEY HELE and NORMAN WINGATE PIRIE.

From the Biochemical Laboratory, Cambridge.

(*Received June 23rd, 1931.*)

The remarkable activity of glutathione *in vitro* and its wide distribution in different animal tissues have, in recent years, focussed attention on its metabolic function. So far however no wholly satisfactory explanation of the high concentration of glutathione in such diverse tissues as liver, lens, and testis has been put forward. The possibility therefore suggests itself that it is a metabolic end-product and that its occurrence is due to an animal's inability to decompose it. A comparison of the behaviour of glutathione and cystine when administered to a dog should throw light on this question since the latter substance is only present in traces, if at all, in such fresh, non-pathological, tissues as have been studied. Some difference in the degree of oxidation was to be expected since Abderhalden and Samuely [1905] have found that less than half of the sulphur given to a dog as dialanylcystine or dileucylcystine is excreted as sulphate. We find however that there is no difference between the behaviour of glutathione and that of cystine in the dog. Of all the cystine-containing peptides which have been studied glutathione is the most completely oxidised[1].

Experimental.

The methods employed in this work were identical with those already described in the earlier papers of this series [Hele, 1924]. Three bitches were used and they received the following diets, half being given in the morning and half in the afternoon. They were catheterised at the same hour each morning and fed immediately afterwards.

	Lean meat	Sugar	Margarine	Milk	Water
	g.	g.	g.	cc.	cc.
Perky (10·5 kg.)	80	70	20	160	100
Violet (10·5 kg.)	80	70	20	160	100
Patsy (8·2 kg.)	80	50	20	160	100

When given orally the substances studied were put in small gelatin capsules which were wrapped up in a thin slice of meat (part of the diet) and when injected they were dissolved in 5–10 cc. of water containing, in the case of acids, the amount of sodium hydroxide necessary to bring the p_H to about 7.

[1] It has been shown in some unpublished work by Hele, Hopkins, Lawrie, Leese, Meldrum and Pirie that a cystinuric could oxidise 3 g. of glutathione, given by mouth, as readily as cystine.

On two of the dogs we tested the experimental technique by injecting subcutaneously 3·22 g. of sodium sulphate (decahydrate) dissolved in 10 cc. of water. The "extra" sulphate in the urine on the following day was 98 % of the dose in each case.

Cysteine hydrochloride and *l*-cystine were prepared from human hair in the usual way and *r*-cystine was prepared from the latter by the method of Hoffman and Gortner [1922]. *r*-Cysteine hydrochloride was made by reducing the *r*-cystine with tin and hydrochloric acid. The methods of preparation of acetylcysteine, chloroacetylcysteine, dichloroacetylcystine, glycylcysteine and oxidised glutathione have already been described [Pirie, 1931] as has the method of preparation of reduced glutathione [Pirie, 1930].

These substances are not wholly without ill effect on the dogs. Unless the dose is fed in two portions some hours apart the acidic compounds occasionally cause vomiting and on the occasion when oxidised glutatione was injected the dog had a mild attack of diarrhoea. Experiments involving any alimentary disturbance have been excluded from Table I except in the case of oxidised glutathione given subcutaneously.

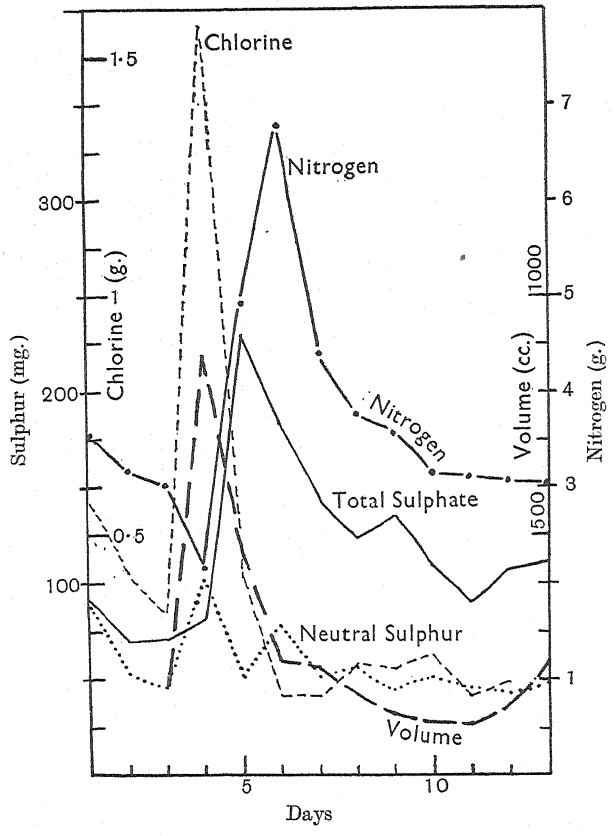


Fig. 1. Perky: 0·16 g. of glycylcysteine anhydride given subcutaneously on day 3. 0·5 g. of glycylglycine anhydride given subcutaneously on day 10.

Once the feeding of 1·2 g. of cystine on a low protein diet (*i.e.* meat 10 g., sugar 60 g., margarine 40 g., milk 50 cc., water 100 cc.) caused haematuria, but this did not happen on four other occasions although there was always a great diminution in the volume of urine passed on the day after the dose.

We attempted to study the metabolism of glycylcysteine anhydride prepared by the method of Hopkins [1929]. 1·2 g. was fed to Patsy on December 9th, she had a marked diuresis on the 11th, 12th, 13th and 14th; the daily volume of urine increased from about 210 cc. to 420 cc. and, since her fluid intake remained constant, she lost 600 g. weight. Her nitrogen and sulphur excretions were very erratic. In an experiment on Perky (Fig. 1) 0·16 g. of glycylcysteine anhydride was given subcutaneously on the 3rd day. The diuretic action and general metabolic upset are clearly shown in the graph. In the urine there were blood cells but no casts and the delayed excretion of

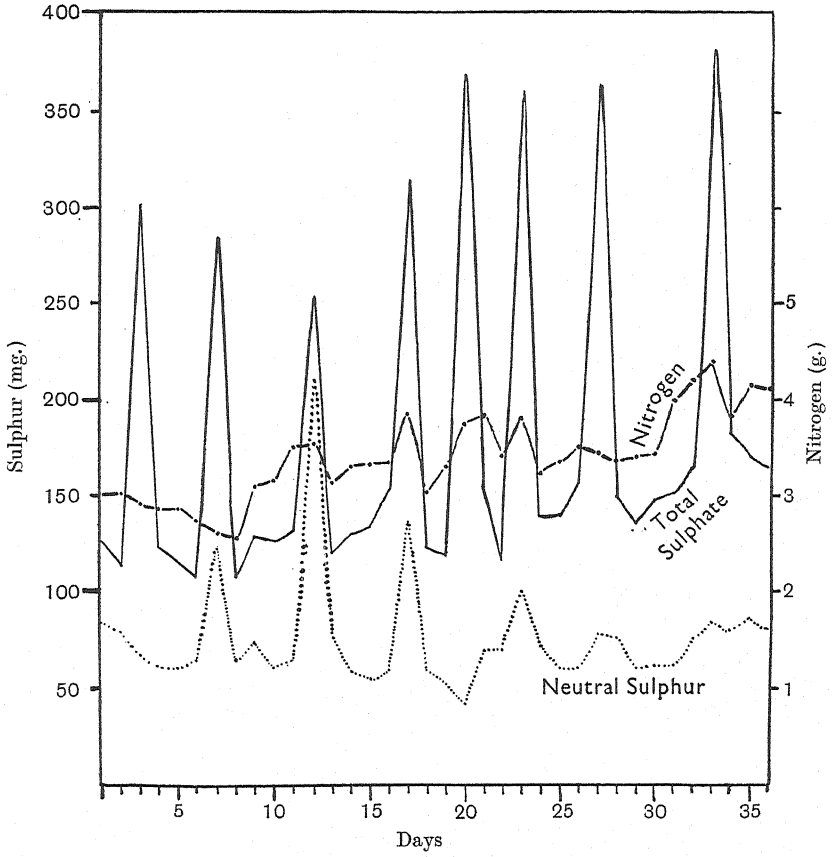


Fig. 2. Patsy: day 3. 1/100 g. mol. cysteine hydrochloride *sub cutem.*
7. 1/100 glycylcysteine *sub cutem.*
12. 1/100 acetylcysteine *sub cutem.*
17. 1/200 oxidised gluthathione *sub cutem* (see note, Table I).
20. 1/200 ,, ,, *per os.*
23. 1/100 *r*-cysteine hydrochloride *per os.*
27. 1/100 glycyl cysteine *per os.*
33. 1/200 cystine *per os.*

nitrogen and sulphur shows that there was some alteration in the permeability of the kidney. On the 10th day she received 0·5 g. of glycylglycine anhydride subcutaneously without any such effect. The oral administration of 0·3 g. of diglycylcystine dianhydride had a similar, but smaller, diuretic action while 1·2 g. of glycylglycine anhydride had none. Four rats, which had each received 6 mg. of glycylcysteine anhydride, all showed a similar diuresis, but even 0·1 g. was without effect on each of three rabbits whether they were fed on a diet of cabbage or on one of bread and milk. Lewis and Lewis [1927] found that dialanylcystine dianhydride was absorbed by a rat and Brand and Sandberg [1926] injected 70 mg. into a rabbit. In neither paper is any ill effect mentioned. Abderhalden and Wacker [1908] fed 10 g. of glycylglycine anhydride

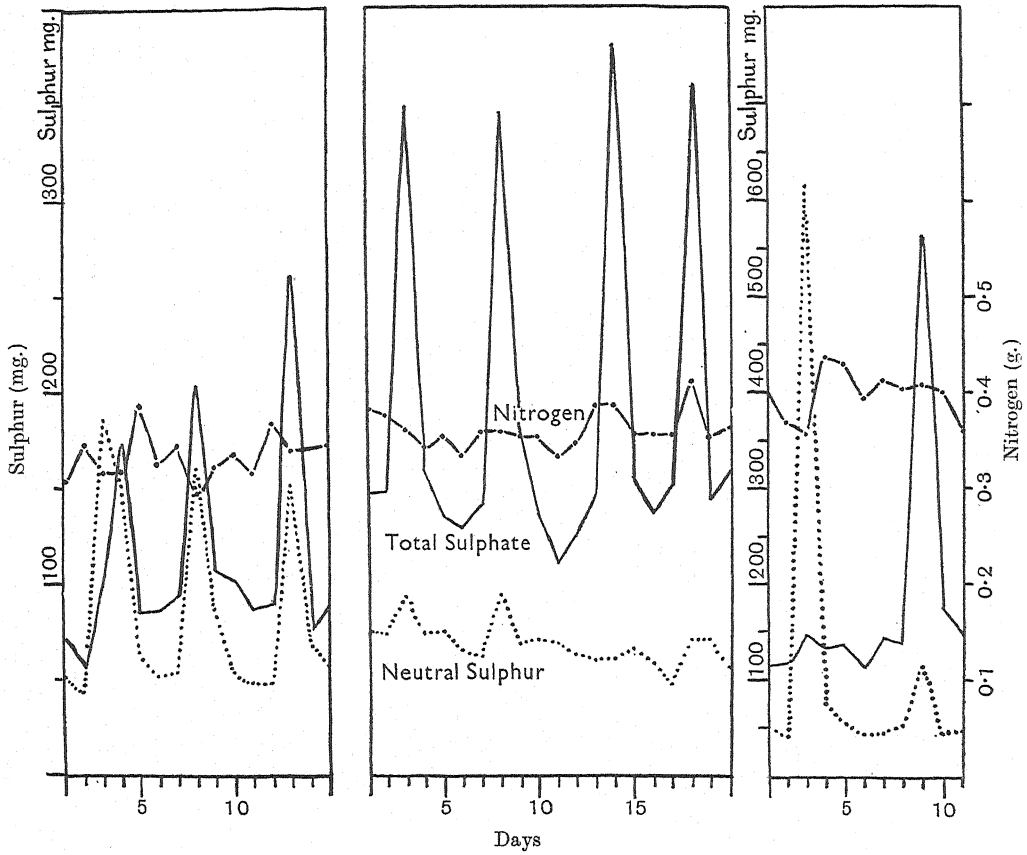


Fig. 3. Violet: day 3. 1/200 gm. mol. dichloroacetylcystine *per os.*
8. 1/100 acetylcysteine *per os.*
13. 1/100 *r*-cysteine hydrochloride *sub cutem.*

3. 1/100 cystine *per os.*
8. 1/100 *r*-cystine *per os.*
14. 1/100 reduced glutathione *per os.*
18. 1/100 „ „ *sub cutem.*

3. 1/100 dichloroacetylcystine *sub cutem.*
9. 2/100 cysteine hydrochloride *sub cutem.*

to a rabbit and mention a diuretic effect; they also found that most of it was absorbed. It is clear however that the diuresis caused by glycylcysteine anhydride is of an entirely different order from that due to glycylglycine anhydride. In spite of its fairly high solubility only a small part of the glycylcysteine anhydride which we fed to a dog seemed to be absorbed, and only 140 mg. (12 % of the dose) could be recovered from Patsy's urine although such a compound is relatively easy to isolate. This amount corresponded to the rise in neutral sulphur.

Only one experiment was carried out with *p*-iodophenylcysteine [Baumann and Schmitz, 1895] since 1·6 g. given orally made the dog vomit, and iodine estimations on the urine, vomit and faeces showed that less than 5 % of the dose had been absorbed.

Analytical data for some of the experiments are shown graphically in Figs. 2 and 3. In all cases there were two or three more blank days at the beginning and end of each set of experiments than are shown. The course of the experiments of which the results only are given was similar.

Table I.

Substance	Dog	Mg. S given	How given	% excreted as sulphate: 1st day	2nd day	Total	% excreted as neutral sulphur: 1st day	2nd day	Total	% accounted for
l-Cystine	P	320	Orally	66	7	73	0	0	0	73
,,	P.	160	,,	74	0	74	0	0	0	74
,,	V.	320	,,	70	0	70	7	0	7	77
,,	V.	320	,,	61	14	75	0	0	0	75
,, *	P.	320	,,	31	31	62	4	7	11	73
,, *	P.	320	,,	69	6	75	6	0	6	81
,, *	V.	320	,,	41	22	63	—	—	—	—
,, *	V.	640	,,	48	24	72	—	—	—	—
r-Cystine	V.	320	,,	67	13	80	9	0	9	89
d-Cysteine-HCl	V.	640	Subcutaneously	66	4	70	10	0	10	80
,,	P.	320	,,	58	3	61	0	0	0	61
,, †	V.	320	Orally	69	3	72	0	0	0	72
r-Cysteine-HCl	V.	320	Subcutaneously	54	0	54	31	4	35	89
,,	P.	320	Orally	72	0	72	10	0	10	82
,,	Pk.	320	,,	56	4	60	19	0	19	79
,, †	V.	320	,,	57	7	64	8	0	0	72
Acetylcysteine	P.	320	Subcutaneously	40	0	40	46	5	51	91
,,	V.	320	Orally	34	4	38	33	10	43	81
,,	Pk.	320	,,	50	0	50	28	0	28	78
Dichloroacetylcystine	V.	320	,,	14	26	40	42	30	72	112
,,	V.	640	Subcutaneously	6	3	9	89	3	92	101
Chloroacetylcysteine†	Pk.	240	Orally	17	0	17	75	5	80	97
Glycylcysteine	V.	288	Subcutaneously	43	4	47	9	3	12	59
,,	P.	320	,,	56	0	56	18	0	18	74
,,	P.	320	Orally	66	0	66	6	5	11	77
,,	V.	320	Subcutaneously	57	0	57	25	12	37	94
Reduced glutathione	V.	320	Orally	72	6	78	0	0	0	78
,,	V.	320	Subcutaneously	66	0	66	7	6	13	79
Oxidised glutathione‡	P.	320	,,	56	0	56	24	0	24	80
,,	P.	320	Orally	77	11	88	0	5	5	93

* With "low protein" diet. † Given in two doses.
‡ Diarrhoea, urine contaminated.

Discussion.

The work of Abderhalden and Samuely [1905] on the sulphur metabolism of dogs may be summarised as follows.

Substance	Amount given per day (g.)	Number of days	% in urine as sulphate	% in urine as neutral sulphur
Cystine (orally)	1·0	10	65	19
Dialanylcystine (O)	1·6	8	58	14
,, (subcutaneously)	1·6	5	38	24
Dileucylcystine (C)	1·9	2	33	29

Stearns and Lewis [1930] found that rabbits oxidised 92·8 % of a dose of cystine ethyl ester hydrochloride to sulphate in 2 days. We are aware of no other quantitative work on the metabolism of sulphur compounds of this type except the very thorough investigations by Lewis and co-workers and Sherwin and co-workers on derivatives of cystine containing an aromatic group. For our present purpose, on account of the aromatic group, that work is not strictly relevant.

It is clear from Table I that the extent to which a substance (except dichloroacetylcystine) is oxidised is very variable but is much the same whether it is given in the sulphydryl or disulphide form and whether it is given subcutaneously or orally. Our results may therefore be summarised as follows:

Substance	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Cystine and cysteine	69·7	3·7
Racemic cystine and cysteine	66·0	16·2
Acetylcysteine	42·7	40·7
Chloroacetylcysteine and dichloroacetylcystine	22·0	81·3
Glycylcysteine	56·5	19·5
Glutathione	72·0	10·5

The similarity between the figures for acetylcysteine, dialanylcystine, dileucylcystine and, to a lesser extent, glycylcysteine is interesting when compared with the relatively much greater extent to which cystine and its ester and glutathione are oxidised. It has now been shown definitely by Nicolet [1930] and by Kendall and co-workers [1930] that glutathione, as was thought, though on somewhat inadequate evidence [Pirie and Pinhey, 1929], in this laboratory, is glutamylcysteinylglycine. A study of the oxidation of the related peptides glutamylcysteine and cysteinylglycine would be interesting.

It is clear from the figures given that a dog can oxidise *r*-cystine to much the same extent as *l*-cystine; it appears that a greater proportion of the racemic compound is excreted as neutral sulphur. In no case has the nature of this neutral sulphur been investigated but we intend to do this at a later date. The percentage of the sulphur administered which is accounted for is greater in the case of inactive cystine or cysteine than in that of the normal

isomeride. This suggests that, as would be expected, the unnatural isomeride is not used to replace tissue breakdown.

The marked difference between the extent to which dichloroacetylcystine is oxidised when given orally and when given subcutaneously suggests that the substance is only oxidised after hydrolysis. In addition to oral administration chloroacetylcysteine was given subcutaneously after neutralisation. There was no rise in sulphate excretion but 84 % of the sulphur in the dose appeared as neutral sulphur. 2 mols. of sodium hydroxide are necessary to raise the p_H of a solution containing 1 mol. of chloroacetylcysteine to 7. The neutral solution contains chlorine ions, no amino-group is present and the sulphydryl group is unaffected. Presumably either hydroxyacetylcysteine or its anhydride is formed but this has not yet been demonstrated conclusively.

Summary.

The capacity of the dog to oxidise cystine and allied compounds, given orally or subcutaneously, has been studied.

The dog can oxidise to the same degree, *l*- and *r*-cystine and *l*- and *r*-cysteine and glutathione.

Acetylcysteine and glycylcysteine are not so readily oxidised.

Chloroacetylcysteine and dichloroacetylcystine are very resistant.

The nature of the neutral sulphur excreted as result of administering certain of the compounds has not yet been investigated.

The administration of glycylcysteine anhydride causes diuresis in dogs and rats but not in rabbits.

p-Iodophenylcysteine upsets the dog and is not well absorbed.

We thank the Government Grant Committee of the Royal Society for grants in aid of this research.

REFERENCES.

Abderhalden and Samuely (1905). *Z. physiol. Chem.* **46**, 187.
—— and Wacker (1908). *Z. physiol. Chem.* **57**, 325.
Baumann and Schmitz (1895). *Z. physiol. Chem.* **20**, 589.
Brand and Sandberg (1926). *J. Biol. Chem.* **70**, 381.
Hele (1924). *Biochem. J.* **18**, 586.
Hoffmann and Gortner (1922). *J. Amer. Chem. Soc.* **44**, 341.
Hopkins (1929). *J. Biol. Chem.* **84**, 312.
Kendall, Mason and McKenzie (1930). *J. Biol. Chem.* **88**, 409.
Lewis and Lewis (1927). *J. Biol. Chem.* **73**, 535.
Nicolet (1930). *J. Biol. Chem.* **88**, 389.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1931). *Biochem. J.* **25**, 614.
—— and Pinhey (1929). *J. Biol. Chem.* **84**, 321.
Stearns and Lewis (1930). *J. Biol. Chem.* **86**, 93.

[Note added 15. viii. 31.] As the neutral sulphur figure (24 %) in the experiment with injected oxidised glutathione appeared high, we repeated this experiment and certain others on the same dog, Patsy. Stocks being low, we used smaller doses, containing in each case 190 mg. sulphur. The results were as follows: oxidised glutathione given orally; excreted as sulphate 58 % and as neutral sulphur 6 %; given subcutaneously; 50 % and 12 %. Reduced glutathione given orally; 70 % and 0 %. *l*-Cystine given orally; 70 % and 0 %. The high figure in question, therefore, was certainly due to faecal contamination of the urine. The new figures support the argument in the discussion.

CXXVI. THE DETERMINATION OF VITAMIN A IN COD-LIVER OILS (*a*) BIOLOGICALLY, (*b*) CHEMICALLY, (*c*) PHYSICALLY, WITH A STATISTICAL EXAMINATION OF THE RESULTS.

By KATHARINE HOPE COWARD and FREDERICK JOHN DYER, *Pharmaceutical Society of Great Britain*; RICHARD ALAN MORTON, *The University, Liverpool*; and JOHN HENRY GADDUM, *The National Institute for Medical Research.*

(*Received June 26th, 1931.*)

A method for the biological estimation of vitamin A has been described by Coward *et al.* [1930, 1931] which appears to have a greater degree of accuracy than the methods previously used. It therefore appeared desirable to use it for measuring the vitamin A values of several samples of cod-liver oil and to compare these values with the "blue values" obtained by the antimony trichloride (or arsenic trichloride) colour test originally put forward by Rosenheim and Drummond [1925] as a measure of the same factor. Eleven samples of oil and two concentrates were examined:

(*a*) biologically (Pharmaceutical Society);

(*b*) chemically by the colour test ($SbCl_3$) on (1) the oils and (2) their unsaponifiable fractions (Pharmaceutical Society—by F. J. Dyer);

(*c*) physically—the intensity of the 328 $m\mu$ absorption and the intensities of the 572 $m\mu$ and 606 $m\mu$ bands of the blue colour obtained with $SbCl_3$ on the oils (The University, Liverpool—by R. A. Morton).

The results were examined statistically by J. H. Gaddum of the National Institute for Medical Research.

The oils and concentrates which were examined by the three methods are given in Table I.

It is evident from Table VII in which the various results have been collected that, in general, an oil with a high biological value has also the following properties.

(*a*) A high blue value obtained by the action of $SbCl_3$ on the oil itself, though the two values are by no means parallel.

(*b*) A high blue value obtained by the action of $SbCl_3$ on the unsaponifiable fraction of the oil. These values are almost parallel.

(*c*) A high value for the absorption bands 572 $m\mu$ and 606 $m\mu$ obtained by the action of $SbCl_3$ on the oil. The intensities of these two bands run parallel in these oils, though Morton has examined numerous oils in which they do not.

Table I.

Designation	Source	Quality	Date of catch
A	Scotch	Finest non-freezing	1928
B	—	Cattle and poultry	1930
C	Norwegian	Finest non-freezing	1930
D	Icelandic*	Finest non-freezing	1930
E	Icelandic*	Finest non-freezing	1930
F	Icelandic*	Unrefined	1930
G	—	Unrefined (?)	1930 (?)
H	Norwegian	Finest non-freezing	1926
J	—	Cod, suspected of being adulterated with shark-liver oil	1928
K	Scotch and Norwegian (mixed)	Finest non-freezing	1930
L	—	Suspected of being adulterated (Unsap. M. = 1·9 %) (F.F.A. = 4·1 %) (I.V. = 152)	1930
M	Concentrate		
N	Concentrate		

* Received direct from the manufacturers, Messrs I. Spencer, Aberdeen.

(*d*) A high value for the intensity of the 328 $m\mu$ absorption band of the oil itself. These values are almost parallel.

There are, however, certain exceptions to this: *e.g.* oil C has a higher biological value than oil A (about 3 : 2) whereas its chemical and physical values are lower than those of oil A (average about 3 : 4); similarly oil K has a biological value about 2·5 times that of oil A and its chemical and physical values are practically equal to those of oil A. It was obviously necessary, therefore, to have the results examined as carefully as possible, partly to see what faith we could place on the biological tests and then to determine which chemical or physical measurement was generally the truest measure of the vitamin A content. Dr J. H. Gaddum has done this and having calculated the probable range of each test on the severe criterion of $\pm$ twice the standard deviation where $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$, he found that many of the physical measurements lie outside the range of the known error of the biological test and suggested that certain oils might contain substances which were inactive biologically but which might increase the blue value ($SbCl_3$) and the intensity of the absorption band 328 $m\mu$. On the other hand, certain oils might contain physiologically active substances other than vitamin A, or the biological test itself might be subject to unknown errors. On the whole the intensity of the 328 $m\mu$ absorption band gave the best agreement with the biological value, and the blue value obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil was nearly as good. Both of these measures were found to be much more accurate than the Lovibond measure of the blue value obtained with the oil itself.

BIOLOGICAL EXAMINATION OF THE OILS.

The vitamin A value of oil A has been very thoroughly examined and the result described in papers by Coward *et al.* [1930, 1931]. It was found that the relation between the dose of oil given and the mean increase in weight of a group of rats given the daily dose of oil for 5 weeks, after having become steady in weight on a diet deficient in vitamin A, was expressed by the equation:

$$y = 12{\cdot}1 + 40{\cdot}24 \log x,$$

where y equals the mean increase in weight in grams of the group in 5 weeks, and x equals the daily dose of cod-liver oil in mg. This relation held when the numbers of bucks and does in each group were approximately equal. The behaviour of the bucks and does in each group was also calculated separately, and the following equations obtained:

$$y = 11{\cdot}3 + 50{\cdot}3 \log x \text{ (bucks)},$$
$$y = 12{\cdot}4 + 27{\cdot}4 \log x \text{ (does)}.$$

The latter equations have now been used for making comparisons of the biological values of ten samples of cod-liver oil and two concentrates.

Rats were prepared for the test by giving them a diet consisting of:

Caseinogen, light-white (B.D.H.) non-extracted	15 %
Dextrinised rice starch	73 ,,
Dried yeast	8 ,,
Salt mixture (Steenbock's 40)	4 ,,

About 2 units of vitamin D were given to each rat twice a week. Each sample of cod-liver oil was so diluted with olive oil that the required daily dose was always contained in 1 drop of the solution. Daily doses were given by dropping directly into the rat's mouth from tubes selected to deliver not more than 21 mg. and not less than 19 mg. oil in 1 drop. Fresh dilutions were made every 7 days and kept in cold store when not actually in the rat room for feeding. Each sample of cod-liver oil was tested in 1–4 doses. Each dose was tested on large groups of animals (9–26), each animal of a group receiving the same daily dose for 5 weeks. The average increases in weight of the bucks and does of each group were then calculated separately, and, by substitution in the corresponding equation, the dose of oil A which would have brought about this increase in weight was determined. The inverse ratio of this dose to the actual dose of the oil under test is the ratio of the vitamin A contents of the oils. The potency of the fresh oil was then calculated as a percentage of that of oil A. The average of the results from bucks and does on all doses of an oil was taken as the measure of its vitamin A potency. In addition the percentage deviation of each group of bucks and does from the average was calculated. Results are collected in Table II. An analysis of the percentage deviations of the groups reveals a surprising degree of accuracy in the results. Twenty different doses of oils were tested, giving 40 results, as the bucks and does were calculated

Table II. *Biological examination of the oils. The vitamin A value of each oil and concentrate is expressed as a percentage of the vitamin A value of oil A.*

Cod-liver oil	Dose mg.	No. of bucks on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ bucks	Dose of oil A which would bring about this inc. mg.	No. of does on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ does	Dose of oil A which would bring about this inc. mg.	Vitamin A potency (%) relative to oil A according to bucks	Vitamin A potency (%) relative to oil A according to does	Weighted mean	Percentage deviation of each group from the average: bucks	Percentage deviation of each group from the average: does	Date at which each test was begun
B	1·0	10	4·2	10·2	0·723	9	11·8	11·3	0·95	72·3	95	—	− 7·8	+21·2	14. vii. 30
	4·0	4	35·75	12·7	3·06	5	24·6	7·2	2·79	76·5	69·7	—	− 2·4	−11·1	10. vi. 30
										Av. = 78·4		79·7			
C	0·5	7	−0·9	7·5	0·571	10	−0·8	11·6	0·33	114·2	66·0	—	−26·3	−57·4	10. x. 30
	1·0	6	27·7	11·3	2·118	13	20·2	5·1	1·9275	211·8	192·75	—	+36·7	+28·4	25. vii. 30
	5·0	13	61·4	18·3	9·91	4	37·25	11·8	8·075	198·2	161·5	—	+27·9	+ 4·3	10. iv. 30
	5·0	7	54·9	18·2	7·357	11	36·2	11·6	7·393	147·1	147·9	—	− 4·1	− 4·5	20. iii. 30
										Av. = 154·9		156·6			
D	0·5	8	2·5	7·6	0·6685	12	6·33	7·8	0·599	133·7	119·8	—	+ 3·0	− 7·7	21. x. 30
	1·0	8	15·0	13·8	1·185	11	17·0	14·5	1·472	118·5	147·2	—	− 8·7	+13·4	22. viii. 30
										Av. = 129·8		130·1			
E	0·5	10	19·2	12·0	1·436	16	9·4	15·2	0·777	287·2	155·5	—	+63·7	−11·4	23. x. 30
	1·0	10	18·0	11·13	1·358	11	14·9	8·5	1·233	135·8	123·3	—	−22·6	−29·7	17. ix. 30
										Av. = 175·45		171·8			
F	0·1	10	1·7	6·9	0·6442	11	9·8	16·4	0·8037	644·2	803·7	—	+ 2·7	+28·1	26. ix. 30
	0·5	8	40·4	18·4	3·787	12	27·2	8·9	3·4682	757·4	693·6	—	+20·7	+10·6	24. x. 30
	1·0	14	42·4	9·6	4·15	6	30·3	15·7	4·498	415·0	449·8	—	−33·8	−28·3	25. viii. 30
										Av. = 627·3		625·8			
G	1·0	8	18·4	12·2	1·384	8	18·5	4·4	1·671	138·4	167·1	—	− 9·4	+ 9·4	11. ix. 30
										Av. = 152·75		152·75			
H	1·0	10	9·7	12·1	0·929	9	7·0	11·1	0·635	92·9	63·5	—	+18·8	−18·8	25. ix. 30
										Av. = 78·2		78·9			
J	1·0	10	14·6	11·4	1·163	12	15·3	6·9	1·276	116·3	127·6	—	− 4·6	+ 4·6	19. ix. 20
										Av. = 121·95		122·5			
K	1·0	2	31·5	0·5	2·5212	8	22·8	8·72	2·3960	252·1	239·6	—	+ 2·5	− 2·5	4. xi. 30
										Av. = 245·8		247·1			
L	0·067	7	13·7	10·1	1·116	7	12·0	7·6	0·9670	1674·0	1450·5	—	+ 7·1	− 7·1	9. i. 31
										Av. = 1562·25		1562·25			
M	0·02	6	−4·0	17·4	0·49636	4	−2·0	6·96	0·29854	2482·0	1493·0	—	+24·9	−24·9	15. xii. 30
										Av. = 1987·5		2086·4			
N	0·04	5	1·8	6·1	0·6473	5	6·2	1·4	0·5939	1618·25	1484·75	—	+ 4·3	− 4·3	20. i. 31
										Av. = 1551·5		1551·5			

separately. Of these, two results only showed more than 50 % deviation (57·4 and 63·7 respectively) from the average value of the sample of oil; two results showed between 30 and 40 % deviation, eleven between 20 and 30, six between 10 and 20, and nineteen less than 10 %. It is, of course probable that, if more doses of each of the last seven oils or concentrates had been tested, the average would have been somewhat different and some of the percentage deviations correspondingly greater. The results on the whole support our claim that a group of about 10 rats used in a test in this manner will give a result almost always within ± 30 % of the true value. Hence, we claim that the biological values which we have assigned to these oils as the result of this method of testing on much larger numbers of rats must be very near to the true values for the oils.

CHEMICAL EXAMINATION OF THE COD-LIVER OILS.

By F. J. DYER.

The oils used in the biological investigation described above were subjected to the following chemical tests.

A. *The oils themselves.*

The chief values determined were:

(1) antimony trichloride blue value by comparison with Lovibond blue glasses;
(2) natural tint;
(3) acid value;

and the opportunity was taken for carrying out also the following tests:

(4) Fearon's reaction;
(5) phosphomolybdate test;
(6) trichloroacetic acid test.

For suggesting to me these three tests and for kindly advising me concerning the conditions under which he applies them in his own laboratory, I am greatly indebted to Dr O. Rosenheim, of the National Institute for Medical Research.

B. *Unsaponifiable fractions of the oils extracted by the method of Smith and Hazley [1930].*

Antimony trichloride blue value (Lovibond measure) and other tests applied to a chloroform solution of the unsaponifiable fractions.

Experimental.

A. *Examination of the oils.*

(1) *Antimony trichloride blue value (Lovibond).* This determination was made by the Carr and Price [1926] modification of the Rosenheim and Drummond test [1925], standardised to a greater degree of uniformity and precision

by the undermentioned conditions recommended by the Cod-liver Oil Colour Test sub-committee of the Pharmacopoeia Commission [1931]. It is well known that the relation between intensity of colour developed and concentration of an oil is not linear but is represented by a line which falls away at higher concentrations to a curvilinear function. For the final colour values of an oil the concentration was chosen so that readings were made between the Lovibond blue figures 4 and 6, a range over which for most oils intensity of colour produced bears an approximately linear relationship to the concentration. Blue values were, however, taken for other concentrations in order that curves for each oil might be constructed if desired.

The conditions under which the test was carried out were as follows.

Reagent. A solution of antimony trichloride was prepared in pure alcohol-free chloroform, saturated at 20°, and found to contain 23 % $SbCl_3$ immediately before use. It was stored in a stoppered amber-coloured glass bottle.

Procedure. Two grams of oil were weighed into a narrow-necked 10 cc. measuring flask, and the flask filled to the mark with chloroform at 20° and mixed.

0·2 cc. of the solution was delivered from a 1 cc. burette, the graduated portion of which measured 16 cm., into a rectangular cell of 1 cm. in the direction of observation as described below. 2 cc. of antimony trichloride reagent were added, the solutions allowed to mix, and the maximum blue colour was immediately matched in a tintometer by Lovibond glasses. Several readings were taken on each oil to ensure that the maximum intensity had been recorded.

Samples of six of the oils were also distributed to three other laboratories and similar determinations to the above were made under the same conditions.

(2) *Natural tint.* The colour of the oils was considered of importance in view of the statement of Drummond and Hilditch [1930] that "a yellow oil is probably richer in vitamins than one paler in colour" if freshly prepared, and even when old, provided that proper precautions have been taken in the making to remove all traces of liver tissue and accompanying enzymes.

The natural tint was determined by placing some oil in a tintometer cell measuring 1 cm. in the direction of observation and matching the colour as seen by diffused daylight with Lovibond yellow and red glasses in a tintometer.

(3) *Acid value.* The importance of a medicinal cod-liver oil possessing a low acid value, preferably below 0·3 % of free fatty acids calculated as oleic acid, has been emphasised by Drummond and Hilditch [1930]. It is also of importance in helping to determine how far the yellow tint of the oil may be regarded as significant, for a high acid value would indicate the presence of fat-splitting enzymes in accompanying liver tissue which would darken the oil.

Free acidity was determined in the customary manner by titration of a weighed quantity of oil in hot neutral alcohol with $N/10$ potassium hydroxide

using phenolphthalein (1 % in alcohol) as indicator. The percentage of free fatty acid was calculated as oleic acid.

(4) *Phosphomolybdate test.* When a solution of cod-liver oil in chloroform or light petroleum is shaken with a solution of sodium phosphomolybdate (10 %) in nitric acid (5 parts strong acid with 95 parts water), and, after allowing the mixture to stand, a strong solution of ammonia (sp. gr. 0·880) is added to the upper layer, a blue colour is developed. As the intensity of the colour is influenced by the time of shaking and by the length of the interval before adding the ammonia, the arbitrarily-chosen conditions set out below were adopted for each oil examined.

Method. To 1 cc. of 20 % solution of cod-liver oil in chloroform (or such other concentration as to give readings between 4–6 blue units as before), contained in a 10 cc. stoppered cylinder were added 2 cc. reagent. The mixture was shaken for exactly 10 seconds, allowed to stand for 1 minute, and then 3 cc. strong ammonia added and allowed to mix with the upper green layer. The maximum blue colour produced was read in a Lovibond tintometer in a cell measuring 1 cm. in the direction of observation using diffused daylight.

There is no evidence that the colour produced in this reaction bears any relation to vitamin activity, but it was thought to be worth while to determine whether there existed any relation between this chromogenic substance and the intensity of blue colour produced by the same oil with antimony trichloride.

(5) *Trichloroacetic acid test.* When trichloroacetic acid is shaken with a chloroform solution of cod-liver oil or preferably with a solution of its unsaponifiable matter, a blue colour is produced, and it was thought to be of interest to compare the intensities of the colours given under standard conditions by the cod-liver oils under investigation. The reagent for the test was prepared by mixing together:

Trichloroacetic acid (A.R.)	100 g.
Distilled water	10 cc.

allowing to stand overnight and using the resulting clear solution. The blue colour produced was measured in exactly the same manner as that described under the antimony trichloride test, using 2 cc. reagent and 0·2 cc. of a solution of the oil in chloroform of such strength that the range of Lovibond blue colour was between 4 and 6. It was thought that this colour, which is usually intensified after saponification of the oil, might bear some relationship to the antimony trichloride blue colour.

(6) *Fearon's Reaction.* Fearon [1925] using the converse of a test devised by Whitby [1923] for detecting sterols, claimed that the rose-pink colour produced in the presence of a condensing agent by the addition of a polyphenol, such as pyrogallol or resorcinol, to a chloroform solution of cod-liver oil was a measure of vitamin A potency. This claim was refuted by Rosenheim and Webster [1926], who showed that unsaponifiable matter of cod-liver oils in which vitamin A was abundantly present failed to give Fearon's reaction.

Further, they regenerated the chromogenic substance from the lead soaps of the unsaturated fatty acids of the original oil, and from other evidence suggested that the chromogen was probably an aldehydic oxidation product of an unsaturated acid (*e.g.* of the clupanodonic series). It was, therefore, considered of interest to apply the test to the oils under examination to see whether any relationship existed between this chromogen and the unsaturated fatty acids to which Norris and Church [1930] and others attribute interference with the blue colour produced by antimony trichloride with cod-liver oils at higher concentrations.

The method used was as follows.

To 0·5 cc. of 20 % solution of cod-liver oil in chloroform contained in a 10 cc. graduated stoppered cylinder was added sufficient trichloroacetic acid solution (12 % in light petroleum) to make 5 cc. A few crystals of pyrogallol were added and the mixture well shaken at intervals during 30 minutes. Another similar cylinder was prepared, containing in addition to the above mixture 3 drops (delivered from a standard dropper and each weighing 0·02 g.) of 1 % solution of purified benzoyl peroxide. (Commercial benzoyl peroxide was dissolved in chloroform and precipitated by methyl alcohol.) The rose pink colour produced was measured, using diffused daylight in a Lovibond tintometer, the mixture being contained in a cell measuring 1 cm. in the direction of observation. Any difference between the tints with and without added peroxide may give some indication of the peroxide content of the oil. The reagent was prepared by diluting with three parts of light petroleum a saturated solution of trichloroacetic acid (A.R.) in light petroleum, the solvent being previously dried over concentrated sulphuric acid as suggested by Fearon.

Results.

The values obtained by the above investigation are summarised in Table III. It may be seen that none of the determinations by the phosphomolybdate test, the trichloroacetic acid test or Fearon's reaction can be considered as a measure of the substance measured by the antimony chloride test.

In Table IV are the results obtained by four different laboratories for six of the oils examined by the antimony trichloride test under rigid conditions to ensure as close uniformity as possible. Although their individual results differ widely, the order of potency of the oils (Table IV), obtained by each of the four workers, is almost the same except for oils B and H. At another time by special request, one of these workers (N. E.) examined oil F and recorded a blue value between 70 and 90, which is in close agreement with the writer's finding of 86.

B. *Preparation and examination of the unsaponifiable fractions of these oils.*

The unsaponifiable fractions of the oils were prepared by the rapid method described by Smith and Hazley [1930], in which 2 g. of the oil are saponified

by 10*N* potassium hydroxide in an open tube, and the unsaponifiable residue is extracted by repeated shakings with chloroform until the chloroform washings from the soap solution no longer give a blue colour with arsenic trichloride.

The supply of oils D, E and F was not sufficient to admit of their unsaponifiable residues being prepared.

The technique was slightly modified by the addition of a few drops of alcohol to the second and third extractions to effect more rapid separation of the mixture into two layers.

(1) *Antimony trichloride blue value.* The residues obtained by evaporation of the mixed chloroform extracts in the above method were dissolved in chloroform to a volume 5, 10 or more cc. according to the known blue value of the oil under examination. The proportion of unsaponifiable matter was not determined, but the results given in Table V are expressed as values of unsaponifiable matter equivalent to the 2 g. of original oil used in its preparation. As a rule, the results show that when readings are taken at the 4 to 6 range of Lovibond glasses and converted to the 20 % concentration, the unsaponifiable matter gives a higher blue value than the amount of oil from which it was prepared. This is in accord with the results of Smith and Hazley.

It may be further observed that whereas for oils of lower antimony trichloride values the relationships to oil A of their blue values after saponification have not greatly altered from the same relationships before saponification, this does not hold true for oils such as F and L of higher blue values. Apparently the somewhat drastic method of saponification has caused considerably more loss of chromogen in these oils than in other oils.

(2) *Fearon's reaction.* Concentrates of some oils unexpectedly gave traces of the Fearon reaction, which appears to show that by this method, saponification is not always complete. No consistent parallelism between Fearon-values

Table III. *Constants and colour values of cod-liver oils used in the biological investigation.*

Oil	Natural tint: Lovibond yellow	Natural tint: Lovibond red	Acid value % F.F.A.	Antimony trichloride test. Value calculated for 20 % concentration of oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of oil. Lovibond blue	Fearon's reaction: Without benzoyl peroxide	Fearon's reaction: With benzoyl peroxide
A	1·8	0	0·34	9·0	1·5	2·7	0·5	0·7
B	3·5	0·3	2·27	4·4	4·7	Nil	12·0	12·5
C	1·2	0·2	0·17	7·6	Trace	1·0	0·4	5·5
D	2·5	0·2	0·38	10·8	—	—	—	—
E	1·6	0·1	0·2	15·2	—	—	—	—
F	8·9	0·8	2·89	86·0	—	—	—	—
G	2·4	0·3	0·38	28·0	Trace	3·9	6·7	11·2
H	1·3	0·1	0·24	4·3	2·3	Trace	2·1	2·3
J	9·2	1·2	1·54	17·4	Trace	2·8	11·7	21·1
K	1·2	0	0·28	10·2	3·5	1·5	2·2	4·0
L	12·0	1·4	4·0	305	Nil	104	Nil	Nil

and blue values obtained by other tests, has been detected during this investigation.

(3) *Trichloroacetic acid test.* One feature of incidental interest is that the blue value given by trichloroacetic acid is greater for a concentrate than for the amount of oil from which it was prepared (Tables III and V). It is possible that an interfering substance has been removed during saponification and that thereby the chromogen is enabled to exert its full power. For concentrates A, B, C, G and H it will be seen that the blue values given by the trichloroacetic acid test run roughly parallel with values given by those concentrates with antimony trichloride.

Table IV. *Antimony trichloride Lovibond blue values of six of the cod-liver oils obtained by four different workers. (Calculated for 20 % dilutions of the oil.)*

Oil	H. A. J.	N. E.	T. T. C.	F. J. D.
A	11·2	11·1	12·4	9·0
B	4·4	4·4	4·7	4·4
C	8·1	8·0	10·0	7·6
G	24·0	24·0	30·6	28·0
H	5·7	5·1	4·1	4·3
J	16·0	13·8	24·0	17·4

It will be seen that although there is a wide variation in these figures, there is a fairly good agreement as to the ascending order of potency, which may be expressed thus:

Order of potency (Lovibond blue) of the six oils according to:

H. A. J.	B H C A J G
N. E.	B H C A J G
T. T. C.	H B C A J G
F. J. D.	H B C A J G

Table V. *Colour values given by unsaponifiable residues of cod-liver oils of Table III, and by two concentrates.*

Note. The unsaponifiable residues were prepared by the method of Smith and Hazley using 2 g. of oil.

Oil	Antimony trichloride test. Value calculated for 20 % concentration of original oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of original oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of original oil. Lovibond blue	Fearon's test (with added benzoyl peroxide). *Note.* For unsaponifiable residues this should be negative (see text). Lovibond red
A	17·25	1·7	8·5	1·6
B	8·0	8·3	4·2	1·2
C	12·9	1·2	5·3	1·0
D	21·2	—	—	—
E	27·0	—	—	—
F	100·0	—	—	—
G	42·0	2·0	24·6	1·2
H	12·6	1·4	7·2	1·0
J	26·6	1·0	8·8	Nil
K	19·6	2·1	17·2	Trace
L	266·0	Nil	230·0	Nil
M	420·0	—	—	—
N	290·0	—	—	—

PHYSICAL EXAMINATION OF THE OILS.

By R. A. MORTON.

All the materials tested biologically have been examined as carefully as possible by the available physical methods. The spectroscopic measurements on the blue colours were carried out on the reagent as described by Carr and Price. The investigation of ultra-violet absorption followed the technique described by Morton and Heilbron [1928]. No corrections have been introduced. The notation $E^{1\%}_{1\,\text{cm.}}$ refers to the intensity of absorption given by a 1 % solution of the material to be tested using a 1 cm. cell, the concentration referring to the actual solution studied in the spectroscope. E is defined by $\log I_0/I = E$, I_0 being the intensity of the incident light and I that of the emergent light (entering and leaving the absorption cell).

It must be emphasised that, since the absorption at 328 $m\mu$ is not wholly due to one substance, the values for $E^{1\%}_{1\,\text{cm.}}$ are all likely to be high compared with the intrinsic absorption of vitamin A (if it proves to be such). The correction to be applied is, however, very small in some fresh cod-liver oils but must differ from oil to oil. In concentrates the magnitude of the correction remains equally uncertain. Instead, therefore, of introducing any corrections we have used the gross intensity of absorption at 328 $m\mu$. The instrumental error is about $\pm$ 3 % in this work, but the uncertainty as to the magnitude of the correction may raise the gross experimental error in extreme cases to $\pm$ 10 %.

With regard to the spectroscopic measurement of the absorption at 572 and 606 $m\mu$ in the blue solutions, it must be emphasised that none of the oils was very fresh when received for spectroscopic examination. The 606 $m\mu$ chromogen (arising in part from the spontaneous oxidation of a substance occurring in many oils [Heilbron, Gillam and Morton, 1931]) was always present in reasonable quantity, so that the 572 $m\mu$ absorption could not be seen visually in the spectroscope as a definite band. The readings were made by setting the spectroscope to 572 $m\mu$ and determining the intensity of absorption irrespective of whether a clear absorption band could be seen or not. There is almost certainly some degree of overlapping between the two absorption bands with their maxima at 572 $m\mu$ and 606 $m\mu$ respectively. The values for $E^{1\%}_{1\,\text{cm.}}$ 572 $m\mu$, therefore, may require some correction, but as we have no exact basis for this the values have not been adjusted. The gross value for $E^{1\%}_{1\,\text{cm.}}$ 572 $m\mu$, does not, however, appear to be very inaccurate; the experimental error may be $\pm$ 10 %.

From other investigations [Gillam and Morton, 1931; Heilbron, Morton and Gillam, 1931] we are quite satisfied that, in extreme cases, the agreement between the 606 $m\mu$ band and either the 572 $m\mu$ or 328 $m\mu$ bands may be very much worse than is indicated by the data published in this paper.

Results.

The results of the physical examination of the oils are summarised in Table VI.

Table VI.

Oil	Date of examination	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ ultra-violet	$E^{1\%}_{1\,cm.}$ 572 $m\mu$ spectroscopic	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ colour test
A	December, 1930	0·78	0·70	0·92
A	April, 1931	0·80	0·75	1·04
B	December, 1930	0·60	0·37	0·47
C	December, 1930	0·67	0·50	0·61
D	December, 1930	0·935	0·92	1·14
D	April, 1931	0·90	0·74	0·95
E	December, 1930	1·03	1·02	1·35
E	April, 1931	1·0	0·80	1·09
F	December, 1930	4·3	3·81	4·76
G	December, 1930	1·68	1·45	1·92
H	December, 1930	0·46	0·33	0·33
J	December, 1930	1·30	1·28	1·50
K	December, 1930	0·85	0·68	0·85
L	April, 1931	8·0	7·5	12·15
M	April, 1931	13·0	15·3 (583 $m\mu$)	21·2 (620 $m\mu$)
N	April, 1931	10·0	9·14 (583 $m\mu$)	11·4 (620 $m\mu$)
Estimated experimental error (gross)		±10 %	±10 %	±10 %

It is clear that a very close agreement occurs between the 328 $m\mu$ absorption and the 572 $m\mu$ absorption. A similar agreement occurs in these samples between the 328 $m\mu$ and the 606 $m\mu$ absorption bands, though the small deviations which exist between the 328 and 606 $m\mu$ bands on the one hand and between the 328 and 572 $m\mu$ bands on the other lie in opposite directions. This agreement between the 328 and 606 $m\mu$ bands is probably misleading, for it has been shown [Gillam and Morton, 1931] that, in many samples of cod-liver oil, there is close agreement between the 328 and 572 $m\mu$ bands but wide disagreement between the 328 and 606 $m\mu$ bands.

STATISTICAL EXAMINATION OF RESULTS.

By J. H. GADDUM.

The results obtained have been examined statistically in order to extract information as to the relative accuracy of the different methods of measuring the concentration of vitamin A, and in order to discover whether the discrepancies shown by some of the oils could be attributed to the known error of sampling of the biological assay.

When the error to be expected in the measurement of any given quantity is proportional to the quantity itself, this error is usually expressed as a percentage of the result. The percentage standard deviation, which is sometimes called the coefficient of variation, is a convenient index of accuracy so long as it is small compared with 100, but when it is large its exact interpretation becomes ambiguous. An error of + 100 % or more may occur in a biological assay, but the expression "an error of − 100 %" has no useful meaning. When

the error is not small compared with 100 % it is clearly not desirable to express it as a percentage at all. Another difficulty arises from the doubt as to whether the error should be calculated as a percentage of the observed value or of the true value, and from the difficulty of carrying out the calculation in the latter case.

These difficulties disappear when the distribution of the logarithms of the readings is studied instead of that of the readings themselves. The error of the test can then be directly expressed as the error of the logarithm of the reading. In the present case the standard deviation of the logarithms (to base 10) of the results has been calculated and the square of this quantity is referred to as the "logarithmic variance." The meaning of the result expressed in this way is not immediately intelligible to those who are not accustomed to this form of expression, but when the calculations are otherwise complete, the result can easily be put in a more readily intelligible form. This method of calculation has been adopted here because, in cases like this, it is simpler, less ambiguous, and probably more rational than the calculation of coefficients of variation. For this purpose it is assumed that the logarithms of the readings are normally distributed. Arguments were put forward by Galton [1879] in favour of the view that, especially in the case of vital phenomena, this assumption was more rational than the usual assumption that the readings themselves were normally distributed. When the error is small compared with the actual readings, the two assumptions give almost identical results, and the chief reason that the former assumption is not generally adopted is probably that, in many cases, it would increase the complexity of the calculations without appreciably affecting the final result [Yule, 1924, Chap. VII, Par. 26].

The sampling error of the biological test.

The sampling error is due to the fact that, even when the conditions are so carefully controlled that the average response of the rats to the vitamin is constant, different individual rats will give different responses. The error is diminished by taking the average response of a number of rats, and its size is dependent on the number of rats used.

The standard deviation (σ) of the response of individual rats (increase of weight) has already been worked out from the results obtained with oil A and published [Coward *et al.* 1930]. These results appear to justify the assumption that this standard deviation is independent of the dose of cod-liver oil used. In order to obtain the best possible estimate of σ the published figures have been recalculated, using the equation $\sigma^2 = \frac{\Sigma d^2}{n-1}$ where $d =$ the deviation of each individual response from the mean response, and $n =$ the number of animals. This formula is likely to give a more accurate estimate than that previously used. The most probable value of σ^2 was found by taking the weighted mean of the different values of σ^2 thus obtained. The weight of each individual estimate of σ^2 was taken to be proportional to n. Separate estimates of the value of σ

for male rats and for female rats have also been obtained by applying the same method to the results with oils B–N. The estimates obtained by these calculations are shown in Table VIII.

It has been found that the response bears a linear relationship to the logarithm of the daily dose. If therefore the distribution of the response is normal, the distribution of the logarithm of the result of the assay will also be normal. A series of biological assays carried out in this way thus probably provides a good example of a series of readings of which the logarithms are normally distributed. This is the kind of distribution implied by the assumptions made in this paper and discussed above.

The standard deviation of the logarithm of the result (λ_1) is obtained by dividing σ by the slope of the line connecting the response with the logarithm (to base 10) of the dose. This is shown in the last column in Table VIII. Since the slope of the curve for male rats is greater than that for female rats, the error of the result is smaller when male rats are used than when female rats are used. It is therefore preferable to use male rats for this test when possible. The largest source of error in these estimates of λ_1 probably lies in our estimate of the slope of the curve connecting dose and effect. The data were considered insufficient for a satisfactory estimate of this error. The error of each test has therefore been calculated on the assumption that $\lambda_1 = 0{\cdot}4$ for all the rats used, and has been taken to be equal to $\frac{0{\cdot}4}{\sqrt{n}}$. It is reasonable to assume that the estimate obtained in this way is larger than the true sampling error of the test. Nevertheless it is found that the errors so calculated are not large enough to account for the discrepancies which occur between physical and biological estimates.

The limits between which the biological result may be expected to vary owing to the error of sampling are shown in Table IX. These limits correspond to twice the standard error; the two values given for each oil were obtained by first dividing and then multiplying the biological estimate by the antilogarithm of $\frac{2\times 0{\cdot}4}{\sqrt{n}}$, where n = the number of rats used. The potency corresponding to the standard curve has been taken as 100. This is probably equal to the potency of the oil A. The error in the estimate of the potency of the oil A from the curve has been calculated in the same way as the errors for the other oils.

The relative accuracy of the different methods of estimating vitamin A.

The results on which these calculations are based are shown in Table VII. The ordinary methods of calculating correlation or regression coefficients are not suitable in this case, because they give undue weight to the results obtained with the more potent oils. The method which has been used depends on calculating the distribution of the logarithms of the results. Some general arguments justifying the assumptions involved have been given.

Let x be any one of the figures in column I of Table VII, and y the corresponding figure in column II. If the two tests both give results directly pro-

Table VII. *Biological, chemical and physical measurements of 11 samples of cod-liver oil and 2 concentrates.*

Oil	Biol. value relative to oil A (=100) I	Blue value, $SbCl_3$ test. Lovibond measure: Oil II	Blue value, $SbCl_3$ test. Lovibond measure: unsap. fraction III	Blue value, $SbCl_3$ test. Spectrophotometric measure: 572 mμ IV	Blue value, $SbCl_3$ test. Spectrophotometric measure: 606 mμ V	Ultraviolet band 328 mμ oil itself VI
A	100	9·0	17·25	0·70	0·92	0·78
B	78·4	4·4	8·0	0·37	0·47	0·60
C	154·9	7·6	12·9	0·50	0·61	0·67
D	129·8	10·8	21·2	0·92	1·14	0·935
E	175·5	15·2	27·0	1·02	1·35	1·03
F	627·3	86·0	100·0	3·81	4·76	4·3
G	152·7	28·0	42·0	1·45	1·92	1·68
H	78·2	4·3	12·6	0·33	0·33	0·46
J	122·0	17·4	26·6	1·28	1·50	1·30
K	245·8	10·2	19·6	0·68	0·85	0·85
L	1562·3	305·0	266·0	7·5	12·15	8·0
M (Conc.)	1987·5	—	420·0	15·3 (583 mμ)	21·2 (620 mμ)	13·0
M (Conc.)	1551·5	—	290·0	9·14 (583 mμ)	11·4 (620 mμ)	10·0

portional to the vitamin A content of the oil, x/y should be constant for the different oils. In order to test how far this is so $(\log_{10}x - \log_{10}y)$ was calculated for each oil. The values obtained, which would be all equal if the error of both tests was zero, were found to vary considerably. The mean and standard deviation of $(\log_{10}x - \log_{10}y)$ were calculated.

Table VIII. *Showing estimates of the standard deviation* (σ) *of the response (increase of weight), and the standard deviation* (λ_1) *of the logarithm of an estimate of the daily dose based on an experiment with one rat.*

	Total number of rats used for the estimate	σ	λ_1
Male and female rats used in experiments with oil A	156	13·2	—
Male rats used in experiments with oils B–N	163	13·4	0·268
Female rats used in experiments with oils B–N	184	10·6	0·386

The mean provides an estimate of the factor by which the blue value of crude oil should be multiplied in order to find the biological activity. This factor has been calculated for all the chemical and physical tests and the results are given in Table IX. In this Table all the different determinations have been reduced to the units in which the biological results are stated by multiplying each figure by the appropriate factor determined in this way. It will be seen that many of the physical measurements lie outside the range of the known error of the biological test. In particular the physical methods appear to have over-estimated the potency of oils G and J and under-estimated those of oils C and K.

If λ = the standard deviation of $(\log_{10}x - \log_{10}y)$, λ^2 gives an estimate of the combined logarithmic variances of the physical and biological methods of estimating vitamin A. If the logarithmic variance of sampling (λ_1^2) represents

Table IX. *Showing the results of the different tests all reduced to the same units and the probable limits of error of the biological test (probability = 21/22). Oils arranged in order of biological activity.*

	Biological value I		Blue value $SbCl_3$ test. Lovibond measure		Blue value $SbCl_3$ test. Spectrophotometric		Ultra-violet band 328 $m\mu$ oil itself
	Most probable	Possible range ($\pm 2\lambda$)	Crude oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	
Oil	I		II	III	IV	V	VI
H	78	51– 119	48	81	57	46	70
B	78	55– 111	49	52	64	65	92
A	100	86– 116	101	111	121	127	120
J	122	82– 181	195	171	221	207	199
D	130	97– 174	121	137	159	157	143
G	153	96– 242	314	271	250	265	257
C	155	125– 193	85	83	86	84	103
E	175	134– 230	170	174	176	186	158
K	246	137– 440	114	126	117	117	130
F	627	495– 794	964	644	656	657	659
N	1551	866–2778	—	1870	1575	1572	1533
L	1562	954–2557	3418	1714	1293	1676	1227
M	1987	1110–3560	—	2170	2637	2924	1993
Factors by which readings in Table VII were multiplied to obtain the above results			11·2	6·442	172·3	137·9	153·3

the whole error of the biological test, the logarithmic variance of the physical test will be equal to $(\lambda^2 - \lambda_1^2)$. This has been calculated for each of the physical tests and the results are shown in Table X. The value taken for λ_1^2 was the

Table X. *Showing estimates of the logarithmic variance of the different tests.*

			Blue value $SbCl_3$ test				
			Lovibond measure		Spectrophotometric measure		Ultra-violet absorption at 328 $m\mu$ of oil itself
		Biological value I	Crude oil II	Unsap. fraction III	572 $m\mu$ IV	606 $m\mu$ V	VI
Biological test	λ_1^2	0·0080	—	—	—	—	—
Ratio of biological to physical results	λ^2	—	0·0554	0·0252	0·0340	0·0328	0·0194
Physical results	$\lambda^2 - \lambda_1^2$	—	0·0474	0·0172	0·0260	0·0248	0·0114
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ (within which the result may be expected to lie 21 times out of 22)		66–151	37–273	55–183	48–210	48–206	61–163

mean of the values for the tests on the different oils. These results have been put in a more familiar form by calculating the range (corresponding to twice the standard error) within which the result may be expected to lie 21 times out of 22, expressed as a percentage of the true value.

The fact that the apparent errors of the physical tests, measured in this

way, are much larger than the errors of the physical measurements themselves might be explained in more than one way. Some oils might contain substances which were biologically inactive and yet gave a blue colour with $SbCl_3$ and absorption in the ultra-violet, while other oils might contain substances which masked the vitamin A in both these physical tests. Both kinds of physical test might measure the concentration of some substance other than vitamin A which has a roughly similar distribution. It is also possible that the biological test is subject to unknown errors due, for example, to a variation in the sensitivity of the whole stock of rats, or to the presence in some oils of physiologically active substances other than vitamin A. It is hoped to test this latter possibility by means of further experiments.

The estimates of the logarithmic variance in Table X place the tests in order of their accuracy when applied to the particular oils used in these experiments, but these estimates are themselves subject to an error of sampling dependent on the number of oils tested. The significance of the difference between these estimates of the accuracy of the different tests has been studied by the methods given by Fisher [1930, Par. 41]. By comparing the estimates of λ^2 it has been found that the probability that the test in column VI is more accurate than that in column II is slightly greater than 0·95. Similar calculations, also based on the estimates of λ^2, show that the probability that the test in column III is more accurate than that in column II is about 0·8.

When allowance is made for the sampling error of the biological test by comparing the values of $(\lambda^2 - \lambda_1^2)$ both these probabilities are greatly increased. The value of λ_1^2 is not known with sufficient accuracy to warrant a precise estimate of the probability, but there is clearly a quite definite presumption in favour of the conclusion that the measurement of the ultra-violet absorption of the oil itself and the measurement of the Lovibond blue value for the unsaponifiable fraction both provide more accurate estimates of vitamin A than the Lovibond blue value for the crude oil.

Discussion.

For practical purposes the possibility of accepting any one of the chemical or physical measurements of the oil as a measure of its vitamin A potency may be determined from an examination of Table XI.

It is evident that the six oils and two concentrates collected in the upper part of Table XI show good agreement between the biological value, the blue value of the unsaponifiable fraction and the intensity of absorption at 328 $m\mu$. Three only of these oils show agreement between biological value and blue value of the oil itself. The five oils collected in the lower part of Table XI cannot be said to show any degree of agreement between biological and physical measurements that would serve any useful purpose, though one of these oils (B) shows better agreement between biological value and the intensity of its absorption at 328 $m\mu$ than between its biological value and any of its other

Table XI.

Oil	Biological value	Blue value of oil	Blue value of unsap. fraction	Intensity of absorption at 328 $m\mu$
H	78	48	81	70
A	100	101	111	120
D	130	121	137	143
E	175	170	174	158
F	627	964	644	659
N	1551	—	1870	1533
L	1562	3418	1714	1227
M	1987	—	2170	1993
B	78	49	52	92
J	122	195	171	199
G	153	314	271	257
C	155	85	83	103
K	246	114	126	130

physical values. It would, therefore, appear to be quite unsafe to accept the blue value of the oil itself as a measure of its vitamin A value. Both the blue value of the unsaponifiable fraction of an oil and the intensity of absorption at 328 $m\mu$ appear to be more often in agreement with the biological value, but these also may be widely divergent, a fact which would appear to indicate that certain oils may contain substances which increase the blue value and other oils may contain substances which decrease the blue value.

The statistical examination of the results has strengthened this opinion.

It may be significant that the oils for which we have found the best agreement between biological and physical measurements are those which we have received direct from manufacturers and which are known to be pure cod-liver oils of recent preparation.

Summary.

Eleven samples of cod-liver oil and two concentrates have been examined and the following values determined.

1. Biological value; vitamin A content as measured by resumption of growth in rats whose reserves of this factor had been exhausted by feeding on a diet deficient in vitamin A.

2. Blue value; obtained by the action of antimony trichloride on the oil itself and measured by the Lovibond tintometer.

3. Blue value; obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil and measured by the Lovibond tintometer.

4. Intensity of absorption at 572 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

5. Intensity of absorption at 606 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

6. Intensity of absorption at 328 $m\mu$ for the oil itself, measured spectrophotometrically.

Statistical examination of the results shows:

(*a*) that the discrepancies between the physical and biological measurements are much larger than the known sampling error of the biological test;

(*b*) that, of the physical and chemical measurements, the best agreement with the figures determined by the biological method was given by the measurement of the 328 $m\mu$ absorption band, and nearly as good agreement was given by the Lovibond blue value of the unsaponifiable fraction of the oil;

(*c*) that both the 572 $m\mu$ and 606 $m\mu$ bands gave less good agreement;

(*d*) that the Lovibond blue values obtained from the oils themselves give the least good agreement and it would appear to be unwise to rely upon this method for the measure of vitamin A in an oil. It can only be used as a very crude indication of vitamin A potency;

The oils whose biological values most closely agree with the values of the 328 $m\mu$ band and the Lovibond blue values of the unsaponifiable fractions are those which were received direct from the manufacturers and examined within a few months of their preparation.

We have to thank Messrs Isaac Spencer and Messrs Allen and Hanbury for supplying samples of oil; Miss B. G. E. Morgan for help given in the biological part of this work and Mr H. W. Ling for help given in the chemical part.

REFERENCES.

Carr and Price (1926). *Biochem. J.* **20**, 497.
Coward, Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
—— —— —— —— (1931). *Biochem. J.* **25**, 551.
Drummond and Hilditch (1930). The relative values of cod-liver oils from various sources, E.M.B. Reports, No. 35.
Fearon (1925). *Biochem. J.* **19**, 888.
Fisher (1930). Statistical methods for research workers. (Oliver and Boyd, London and Edinburgh.)
Galton (1879). *Proc. Roy. Soc. Lond.* **29**, 365.
Gillam and Morton (1931). *Biochem. J.* **25**, 1346.
Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.
Morton and Heilbron (1928). *Biochem. J.* **22**, 987.
Norris and Church (1930). *J. Biol. Chem.* **85**, 477.
Pharmacopoeia Commission Reports of Subcommittees (No. 3) [1931]. Report of cod-liver oil colour test sub-committee, Gen. Med. Council.
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.
—— and Webster (1926). *Biochem. J.* **20**, 1342.
Smith and Hazley (1930). *Biochem. J.* **24**, 1942.
Whitby (1923). *Biochem. J.* **17**, 5.
Yule (1924). An introduction to the theory of statistics. (Charles Griffin and Co., London.)

CXXVII. TRYPANOCIDAL ACTION AND TOXICITY TO ENZYMES.

By JUDA HIRSCH QUASTEL.

From the Biochemical Laboratory, Cardiff City Mental Hospital.

(*Received June 26th, 1931.*)

It has been shown in a previous communication [Quastel, 1931] that the Congo red series of dyestuffs is highly toxic to the enzyme fumarase, prepared either from bacteria or from mammalian tissues. The presence of proteins greatly affects the toxicity of the dyes; this is due to the adsorption or combination of the dyestuff with protein, there resulting a diminution in the concentration of free dyestuff. With fumarase extracted from brain tissue and prepared in such a way as to be relatively free from protein and phosphate, Congo red is toxic at a molar concentration of $1{\cdot}2 \times 10^{-8}$.

Trypan-blue is highly toxic and it has now been found that trypan-red is also toxic, though its activity is not as great as that of the former. It is not to be supposed that these substances are general enzymic poisons, for experiment has shown that neither Congo red nor trypan-blue has any toxic action upon the enzyme urease prepared from Soya bean. It is evident that there must be some close connection between the structure of fumarase and that of the Congo red series of dyestuffs, which allows this specificity of behaviour to obtain.

The fact that trypan-blue and trypan-red have highly toxic actions upon fumarase led the writer to consider the possibility that other trypanocidal agents, not belonging to the Congo red series, might affect the activity of fumarase. Accordingly, Bayer 205 was tested and it has been found that this substance is also very toxic, its behaviour, as in the case of Congo red and trypan-blue, being specific, for it has no toxic effect on urease.

It is necessary now, for a proper appreciation of what follows, to give a brief résumé of recent work on the mechanism of trypanocidal action.

Following the discovery by Ehrlich and Shiga that trypan-red (I),

(I) $C_{10}H_4(SO_3H)_2(NH_2).N:N.C_6H_4.C_6H_3(SO_3H).N:N.C_{10}H_4(NH_2)(SO_3H)_2$,

a member of the Congo red series of cotton dyes, can cure an experimental infection of trypanosomiasis in mice, Nicolle and Mesnil examined a large number of cotton dyes belonging to the Congo red series and found that the substance having the best curative action on infection with *Trypanosoma gambiense* was a *s*-carbamide (II) derived from H-acid.

(II) $C_{10}H_3(SO_3H)_2(NH_2)(OH).N:N.C_6H_4.NH.CO.NH.C_6H_4N:N.C_{10}H_3(SO_3H)_2(NH_2).OH$.

This was followed by the discovery, by the Bayer firm, that the replacement of the azo-linkage in II by the peptide (—NH—CO—) linkage gave rise to a colourless substance also possessing trypanocidal properties.

It was known from work on dyes that the substantive character to cotton was favoured by the presence of chains of aminobenzoyl groups, and, by the introduction of such groups into the *s*-carbamide of an aminobenzoylnaphthylaminepolysulphonic acid, the important trypanocidal agent Bayer 205 (III) was evolved.

(III) $C_{10}H_4(SO_3H)_3NH.CO.C_6H_3(CH_3).NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_3(CH_3).CO.NH.C_{10}H_4(SO_3H)_3$.

As Balaban and King [1927] point out, it is clear that the discovery of Bayer 205 arose from the recognition of the fact that it is possible to prepare colourless substances of substantive properties which, being of similar molecular build to trypanocidal dyes, also exhibit trypanocidal properties.

The next important step was taken by Balaban and King [1927], who started with six readily obtainable naphthylaminedisulphonic acids and from these prepared the *m*-aminobenzoyl and the *m'*-aminobenzoyl-*m*-aminobenzoyl derivatives. They then formed the *s*-carbamides of these derivatives by phosgenation. The *s*-carbamide of the original naphthylaminedisulphonic acid will be referred to as the first *s*-carbamide derivative; that of the *m*-aminobenzoyl derivative as the second *s*-carbamide derivative and that of the *m'*-aminobenzoyl-*m*-aminobenzoyl derivative as the third *s*-carbamide derivative.

Thus:

$NH_2.R \rightarrow R.NH.CO.NH.R$
First *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.R$
m-aminobenzoyl derivative — Second *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_4.CO.NH.R$
m'-aminobenzoyl-*m*-aminobenzoyl derivative — Third *s*-carbamide derivative.

Balaban and King found, on examining the trypanocidal properties of the aminobenzoyl derivatives and of the first, second and third *s*-carbamides of the six naphthylaminedisulphonic acids, that trypanocidal activity only occurred among the second and third *s*-carbamides, *i.e.* at the *s*-carbamide stage of combination of the aminobenzoyl and aminobenzoyl-aminobenzoyl derivatives. Bayer 205, it is to be noted, is a third *s*-carbamide derivative. Following this, these workers then showed, on examining the substantive properties of these substances to cotton, that a pronounced maximum of substantivity occurred at the *s*-carbamide stage of combination and was greater with the third *s*-carbamides than with the second. Thus there appeared to be a definite parallelism between the substantive properties of these carbamides to cotton and their trypanocidal activity. Bayer 205 was also shown to be substantive to cotton.

Turning now to fumarase and knowing that trypan-blue, trypan-red and Bayer 205 were toxic to the enzyme, it became of interest to see whether the *s*-carbamides investigated by Balaban and King had any effect upon the

enzyme. Experiment has shown that toxicity only appears at the second *s*-carbamide stage of combination and that the third *s*-carbamides are highly toxic. The effect, moreover, is specific to fumarase, for urease is unaffected by these derivatives.

Method.

The method adopted throughout this work is that described fully in a previous communication [Quastel, 1931]. It consists, briefly, in mixing a preparation of fumarase, obtained from brain, with phosphate buffer solution, p_H 7·4, and the substance under investigation and in incubating the mixture for 30 min. or 60 min. at 45°. Sodium fumarate solution is then added to the mixture to make a final concentration of 0·08 *M*. Incubation is allowed to proceed for 2 hours at 45°, and the *l*-malate produced is estimated polarimetrically. From a comparison of the *l*-malate produced in presence of the toxic substance with that formed in its absence, an estimate of the inhibiting effect of the toxic agent is obtained. Details of the estimation of the *l*-malate will be found in the previous communication.

The effects of trypan-blue and trypan-red.

To a mixture of 2 cc. fumarase preparation and 1 cc. *M*/5 phosphate buffer, p_H 7·4, was added 1 cc. trypan-blue solution or 1 cc. trypan-red solution, to give a concentration of 1/4000. The mixture was incubated for 30 min. at 45°, and then 1 cc. 0·4 *M* sodium fumarate solution was added. Incubation was carried out for 2 hours at 45°. A mixture of 1 cc. glacial acetic acid and 10 cc. 14·2 % ammonium molybdate solution was then added, and the solution shaken with a little decolorising charcoal and filtered. The filtrate was examined polarimetrically in a 1 dm. tube, compared with a control experiment in which no dyestuff was present, and the amount of inhibition calculated.

The experiment was repeated with various concentrations of the dyes and the results are recorded in Table II. It will be seen that trypan-blue is more toxic than trypan-red but that both are toxic at concentrations of 1/16,000.

The effects of naphthylaminedisulphonic acids.

The following six acids were used:

Freund's acid: 1-naphthylamine-3 : 6-disulphonic acid.
Amino-G-acid: 2-naphthylamine-6 : 8-disulphonic acid.
C-acid: 2-naphthylamine-4 : 8-disulphonic acid.
Amino-J-acid: 2-naphthylamine-5 : 7-disulphonic acid.
H-acid: 8-hydroxy-1-naphthylamine-3 : 6-disulphonic acid.
2R-acid: 8-hydroxy-2-naphthylamine-3 : 6-disulphonic acid.

Experiments were carried out, as described for trypan-blue and trypan-red, but the incubation period prior to the addition of fumarate was one hour and in the estimation of the malic acid it was unnecessary to shake with charcoal.

Results are noted in Table I from which it will be seen that these acids at a concentration of 1/4000 have little or no toxic action on fumarase.

Table I. *Percentage inhibitions of fumarase activity by naphthylaminedisulphonic acids and their* s-*carbamide derivatives. Concentration* = 1/4000.

	Freund's acid	Amino-G-acid	C-acid	Amino-J-acid	H-acid	2R-acid
Free acid	1	0	0	0	3	2
1st *s*-carbamide	—	—	3	4	—	5
2nd *s*-carbamide	5	32	17	19	8	95
3rd *s*-carbamide	83	90	94	95	32	95

The effects of s-*carbamides of naphthylaminedisulphonic acids.*

Specimens of these substances were kindly supplied by Dr H. King. The first *s*-carbamides of Freund's acid, amino-G-acid, and H-acid were not available and were not tested.

Experiments were carried out exactly as with the naphthylaminedisulphonic acids and the complete set of results is given in Table I.

The most striking result is that, whereas the first *s*-carbamide derivatives are practically inert, the third *s*-carbamide derivatives are highly toxic.

There is a great difference in toxicity between the carbamide derivatives of H-acid and 2R-acid, the only chemical difference between these acids being that the former is a 1-naphthylamine derivative and the latter a 2-naphthylamine derivative. The second *s*-carbamide of 2R-acid is as toxic as any of the third *s*-carbamide derivatives of the acids investigated, and far more so than the other second *s*-carbamide derivatives. It would seem that the extra toxicity of 2R-acid is determined by the presence of the hydroxyl group in the naphthalene nucleus and this is supported by the fact that trypan-blue (which contains such a group) is more toxic than trypan-red (from which the hydroxyl group is absent). H-acid, however, which also contains the hydroxyl group in the naphthalene nucleus, forms *s*-carbamides which are not only less toxic than those from 2R-acid but less toxic than those from the non-hydroxylated naphthylaminedisulphonic acids. It is impossible, therefore, to attribute any increased toxicity solely to the hydroxyl group.

It is, however, quite clear that toxicity to the enzyme becomes apparent only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. (Bayer 205 which is a third *s*-carbamide derivative gives a 68 % inhibition of fumarase at a concentration of 1/4000.) Now this is precisely the result obtained by Balaban and King [1927] who found that trypanocidal activity became apparent only at the second *s*-carbamide stage of combination and was most marked at the third *s*-carbamide stage.

It must not be supposed that the toxicity of the higher *s*-carbamides to fumarase is an explanation of their trypanocidal activity, for Balaban and King found that the derivatives of Freund's acid and 2R-acid have no trypanocidal action. It is evident, however, that there must be some structure common to the trypanosome and to fumarase which accounts for the parallelism which

exists between fumarase toxicity and the trypanocidal action of the second or third *s*-carbamides.

Variation in toxicity with concentration of s-*carbamides.*

The experiments described above were repeated with the more toxic *s*-carbamides at various concentrations, the incubation period prior to the addition of fumarate being 30 min. The results are recorded in Table II. The most important result emerging from this Table is that the *s*-carbamide compounds which retain a high toxicity at considerable dilution are all derived from 2-naphthylamine structures. Derivatives of the 1-naphthylamine compounds (Freund's acid and H-acid) have relatively little toxicity at high dilution.

Table II. *Percentage inhibition of fumarase activity by by* s-*carbamides* etc. *at various concentrations.*

	Concentration		
	1/4000	1/8000	1/16,000
Trypan-blue	100	100	92
Trypan-red	74	43	21
3rd *s*-carbamide of Freund's acid	76	52	14
3rd *s*-carbamide of H-acid	29	11	6
2nd *s*-carbamide of amino-G-acid	16	11	3
3rd *s*-carbamide of amino-G-acid	87	78	60
3rd *s*-carbamide of C-acid	97	95	85
3rd *s*-carbamide of amino-J-acid	91	79	45
2nd *s*-carbamide of 2R-acid	93	87	75
3rd *s*-carbamide of 2R-acid	90	80	53

Substantive properties and toxic action.

Balaban and King suggested from their experiments that there is a rough parallelism between the substantivity to cotton and the trypanocidal action *in vivo*, in that the salient peaks of substantivity occurred at the *s*-carbamide stage of combination. They were only able to determine the substantivity of the derivatives of H- and 2R-acids, but since Bayer 205 was also found to be substantive to cotton, it seems likely that the phenomenon holds also for the nonhydroxylated derivatives.

It is obvious that there is also a parallelism between substantivity to cotton and toxicity to fumarase, in that the latter appears only among the second and third *s*-carbamides. The parallelism, however, must be very rough, for whereas the substantive power of the second carbamide of 2R-acid is $2\frac{1}{4}$ times that of the second carbamide of H-acid, the toxic power of the former on fumarase is about 12 times that of the latter.

It is significant in this connection that Congo red and benzopurpurin which are highly toxic to fumarase are substantive to cotton.

On the whole it would seem that the fumarase enzyme, which is very widespread biologically both in plant and animal tissues, must possess structures which are common to the cotton fibre and to the trypanosome and which are responsible for the specific combination or adsorption of the higher

s-carbamide derivatives of the naphthylaminedisulphonic acids. Other factors, specific for the type of biological material, doubtless account for the variations which are observed between the effects of these derivatives on the different biological types.

It is of particular interest that there should be an enzyme to which these trypanocidal agents exhibit a specific toxicity (urease, for instance, is unaffected by them) and the elucidation of the factors involved should be of material assistance in the development of this branch of chemotherapy.

Protection by fumarate.

It has been shown [Quastel, 1931] that fumarase is "protected" by its substrate, fumarate, from such toxic dyestuffs as Congo red and methyl violet. This holds also for the *s*-carbamide derivatives.

To 2 cc. fumarase preparation were added 1 cc. $M/5$ phosphate buffer, p_H 7·4, 1 cc. water, and 1 cc. 0·4 M sodium fumarate solution. After incubation at 45° for 2 hours the rotation was 1·12°.

To 2 cc. fumarase preparation were added 1 cc. $M/5$ phosphate buffer, p_H 7·4, 1 cc. 1/1000 solution of the third *s*-carbamide derivative of 2R-acid, and 1 cc. 0·4 M sodium fumarate solution. After incubation for 2 hours the rotation was 1·03°. (Inhibition = 8 %.)

To 2 cc. fumarase preparation were added 1 cc. $M/5$ phosphate buffer, p_H 7·4, and 1 cc. of 1/1000 solution of the third *s*-carbamide derivative of 2R-acid. The mixture was incubated at 45° for 30 min. and then 1 cc. 0·4 M sodium fumarate solution was added. After incubation at 45° for 2 hours the rotation was 0·11°. (Inhibition = 90 %.)

The experiment shows that incubation with the *s*-carbamide for 30 min. prior to the addition of fumarate gave an inhibition of 90 %, whereas when the derivative was added together with the fumarate the inhibition was only 8 %.

This holds also for other *s*-carbamide derivatives.

Effects of proteins on the toxic action of s-*carbamides.*

As occurs in the case of the toxic dyestuffs, proteins exert a marked protective action against the *s*-carbamides.

This is indicated in Table III where the effects of addition of guinea-pig serum at different concentrations to fumarase are noted.

Table III. *Percentage inhibitions of fumarase by* s-*carbamides* (1/5000) *in presence of serum.*

Concentration of serum	3rd carbamide of amino-J-acid	3rd carbamide of 2R-acid	Bayer 205
1/25	36	40	20
1/50	81	70	33
1/100	89	84	37
1/200	92	91	43
0	92	92	43

A typical experiment was as follows. To 2 cc. fumarase preparation were added 1 cc. $M/5$ phosphate buffer, 1 cc. of diluted guinea-pig serum, and 1 cc.

of 1/1000 solution of the *s*-carbamide. This was allowed to incubate at 45° for 30 min. and then 1 cc. 0·4 *M* sodium fumarate solution was added. After further incubation at 45° for 2 hours the rotation was determined and the percentage inhibition calculated.

There is little doubt that, as in the case of the dyestuffs, the protective action of the protein is due to a combination of the protein with the *s*-carbamide derivative, resulting in a diminution of the concentration of the free derivative and hence in a reduced inhibition of fumarase activity.

SUMMARY.

1. Trypan-blue, trypan-red, and Bayer 205 are toxic to fumarase.

2. The toxicity of six naphthylaminedisulphonic acids and their *s*-carbamide derivatives on fumarase has been investigated. It is shown that the free acids and their first *s*-carbamide derivatives are inert. Toxicity begins to be apparent with the second carbamide derivatives (*s*-carbamide of *m*-aminobenzoylnaphthylaminedisulphonic acid) and is very marked with third carbamide derivatives (*s*-carbamide of *m'*-aminobenzoyl-*m*-aminobenzoylnaphthylaminedisulphonic acid). Attention is drawn to the parallelism which exists between this effect and trypanocidal action which begins only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. The parallelism with the substantive properties of these derivatives to cotton is also discussed, and it is shown that there must be some structure in common between the fumarase enzyme, cotton fibre and the trypanosome which makes for specific combination or adsorption with the second and third *s*-carbamide derivatives of the naphthylaminedisulphonic acids. These derivatives are not toxic to urease.

3. Derivatives of the 2-naphthylaminedisulphonic acids are much more toxic to fumarase than those of the 1-naphthylaminedisulphonic acids.

4. Fumarate "protects" the enzyme, fumarase, from the toxic action of these derivatives.

5. The presence of proteins diminishes or eliminates the toxic action of these derivatives.

My thanks are due to the Medical Research Council for a grant in aid of the equipment of this laboratory and to Dr H. King for supplying me with specimens of the *s*-carbamide derivatives used in this investigation.

REFERENCES

Balaban and King (1927). *J. Chem. Soc.* 3068.
Quastel (1931). *Biochem. J.* **25**, 898.

CXXVIII. THE RELATION OF GLYCOSURIA TO GLYCAEMIA AND THE DETERMINATION OF THE RENAL THRESHOLD FOR GLUCOSE.

By HAROLD PERCIVAL HIMSWORTH.

From the Medical Unit, University College Hospital, London.

(*Received June 27th, 1931.*)

Introduction.

Glycosuria, as the most obtrusive finding in diabetes mellitus, has, since its association with this disease was established, aroused considerable interest both as to its production and as to its significance. Prior to the day of Claude Bernard the presence of sugar in the blood was regarded as denoting an abnormal and definitely pathological condition, and glycosuria was considered to be its attendant, and equally pathological, manifestation. His demonstration that glycaemia was of normal occurrence, and that hyperglycaemia was productive of glycosuria, was the first step towards the modern ideas on the subject, and, in addition, it provided the data upon which Bernard [1877] based his conception of the renal threshold as that level of sugar in the blood at which glycosuria appeared. Some years later the discovery by von Mering [1888] of phlorhidzin diabetes showed that glycosuria could exist apart from hyperglycaemia, but it was only with the realisation that there existed a benign clinical type of this disorder that the idea of the presence of a renal factor in all cases of glycosuria became generally accepted.

Apparently Bernard was of the opinion that when the sugar in the blood exceeded a certain level the kidney permitted sugar to "spill" over into the urine. A definite line could therefore be drawn between glycosuria and aglycosuria. This idea was universally accepted until it was attacked by Benedict, Osterberg and Neuwirth [1918]. They maintain that, in the urine of normal individuals, sugar is always present, and that, after carbohydrate meals, an increase in amount may be detected. They have suggested the term glycuresis for this phenomenon, and claim that there is no such thing as a definite threshold. Folin and Berglund [1922] have criticised this work on the ground that the carbohydrates present in normal urine are not of the nature of glucose, but are unusable carbohydrates which the body naturally excretes. Until glucose has been isolated from normal urine this conception of glycuresis must be considered unproven.

The work of Richards [1929] has produced very strong evidence in favour of the filtration-reabsorption theory of kidney function and considered along

with the recent work of Ni and Rehberg [1930] has thrown considerable light upon the nature of the renal threshold. Richards has demonstrated in frogs the presence of glucose in the glomerular filtrate and its absence from the urine. By studying the blood-sugar and the glycosuria in dogs after the intravenous administration of glucose, in conjunction with Rehberg's creatinine filtrate method of estimating the amount of reabsorbed fluid, Ni and Rehberg have shown that as the blood-sugar rises the amount of glucose reabsorbed by the tubules rises also, but that this increase becomes less and less the higher the level attained by the blood-sugar, and that when the difference in concentration between the sugar in the blood and the sugar in the lumen of the tubules exceeds a certain limit, there is no further increase in reabsorption. It appears therefore that for every concentration of blood-sugar a definite amount of glucose per minute is filtered through the glomerulus, and that also for the same value a definite amount of glucose per minute is absorbed from the lumen of the tubules. Thus there will be one value of the blood-sugar at which the tubules just fail to absorb all the glucose filtered, and glycosuria will occur. This value will correspond to the renal threshold.

A peculiar phenomenon in connection with the threshold was first observed by Frank [1913] and has since been confirmed by many other investigators. Frank found that the level of the blood-sugar at which glycosuria appeared was not the same as that at which it disappeared. In his experience the former was usually the higher, but many cases have been reported in which the latter was the higher. Most observers have been inclined to accept this phenomenon as indicating the presence of two different normal thresholds, but Faber and Hansen [1923] in their review of the subject remark, "we cannot really regard the difference as a sign of change in the threshold; it is a phenomenon by itself which must be separated from the level of the threshold." And up to the present there has been no definite evidence to disprove this statement.

Thus it appears that the balance of evidence is in favour of a renal threshold, or thresholds, of a definite level, and that there is some indirect evidence as to the nature of the underlying mechanism.

Methods previously advocated for the determination of the renal threshold.

When considering the methods which have been recommended for the determination of the renal threshold, one must remember that some of these methods were evolved before much of the now commonplace knowledge of the behaviour of the blood-sugar was discovered, and in assaying any method it is essential to bear the following three facts in mind. First, that the blood-sugar varies considerably throughout the 24 hours according to the food intake; second, that the level of blood-sugar at which glycosuria appears usually differs from the level at which it disappears; and third, that in most,

if not all diabetics, during the night, the blood-sugar does not exhibit a uniform decrease but rises steadily in the early hours of the morning, and then decreases again slowly. This latter phenomenon, noted by Hatlehol [1924], has been confirmed by the writer.

The methods will now be described, and in the consideration of methods (*a*) and (*b*) the debt to the critical review of Faber and Hansen [1923] must be acknowledged.

(*a*) After the night's fast the blood-sugar is estimated, and the urine is tested for the presence or absence of sugar. This procedure is repeated several times during treatment, and from the data thus collected the threshold is adjudged to lie between certain values of the blood-sugar.

Certain workers [Williams and Humphreys, 1919] have based their conclusions upon the presence or absence of sugar in a 24-hour specimen, but in the light of the known variations of the blood-sugar the inaccuracy of such conclusions is obvious. The most accurate modification of the test is that in which the bladder is emptied after a night's fast, urine is passed again a short time later, and the blood-sugar estimated half-way between the two evacuations. From the data thus collected some knowledge of the relationship of the blood-sugar values to the presence or absence of glycosuria may be gathered, but it is obviously impossible by any modification of this test to avoid the discrepancies introduced by the two different thresholds. It is the usual experience with diabetics that as the patient reacts satisfactorily to treatment the value of the morning blood-sugar decreases, and there is a concomitant decrease, and possibly disappearance, of the glycosuria. In a case in which the threshold of appearance is higher than the threshold of disappearance, glycosuria may occur on one occasion and not on another with a blood-sugar of approximately the same value. Failure to take this fact into consideration would account for the common statement that, during treatment, there is a rise in value of the renal threshold.

It appears that this method, and the conclusions drawn from it, have little or no value.

(*b*) After the oral administration of a standard dose of glucose, the blood-sugar is estimated at frequent intervals, and the urine voided at frequent known intervals is tested for sugar. By noting the highest value of the blood-sugar both on the rise and on the fall of the curve, at which the urine is sugar-free, and similarly the minimum value at which glycosuria occurs, an approximate idea of the two thresholds may be obtained.

This method has been used extensively but is open to criticism on the score of the practical difficulties which prevent its accuracy. The frequency with which the bladder can be emptied is limited, and 10-minute specimens are as much as can be expected from an unpractised person. It will easily be seen that, under such natural restrictions, with a rapidly rising or falling blood-sugar curve, considerable error is inevitable, but that on a slowly falling curve a reasonable degree of accuracy can be attained.

Another criticism, which applies equally to all methods involving qualitative testing of the urine for sugar, is the question of the sensitivity of the reagent. The lower limit of sensitivity of Benedict's reagent is in the region of 0·09 % of glucose. Now, if the threshold is exceeded just before a urination the sugar-containing urine then passed will be diluted by the urine already in the urinary tract, and the resulting concentration of sugar may be too small to be detected by the reagent. In cases of renal glycosuria where the concentration of sugar excreted is very low this is a point of considerable importance.

A method similar in principle, and limitations, is that in which the glycosuriac is starved until he is sugar-free, blood-sugar estimations being performed the while. As the slope of such a blood-sugar curve is very gradual, provided one does not meet the nocturnal rise of blood-sugar, one might expect to get a moderately accurate idea of the situation of the threshold of disappearance, but according to Faber and Hansen [1923] this point appears to be lower after starvation than after administration of glucose. Further information is wanted on this point before the factor of experimental error either in this latter method or their control method can be ruled out.

(*c*) On different days graded doses of glucose are given to the patient, the blood-sugar estimated frequently in each experiment, the resulting curve charted, the urine passed frequently and tested for sugar. Curves of varying heights are obtained. The peaks on the lowest curve with glycosuria and the highest curve without are noted and the threshold is said to lie between these two points. The authors of this method, Faber and Hansen [1923], point out that it is essential to estimate the blood-sugar frequently in order to hit the peak of the curve, and also, that it is equally essential to obtain frequent urine specimens as the concentration of the sugar passed, in those experiments near the border line of glycosuria, is so small that dilution renders impossible its detection by the reagent. They claim that it is quite possible, with practice, so to judge the dose of sugar that the peaks of neighbouring curves may be made to lie very close to each other. The reason behind the evolution of this method is to be found in the statement by the above authors quoted previously, namely, that two thresholds do not exist, there being but one threshold which has the apparent phenomenon of the double threshold superimposed upon it. From the results given later two separate thresholds do appear to exist, and, in that case, their figures will correspond to determinations of the threshold of appearance.

(*d*) By a modification of Ambard's formula, Chabanier, Lebert and Lobo-Onell [1929] have produced an equation expressing a theoretical relationship between blood- and urine-sugars and the renal threshold. Their results and conclusions are so much at variance with the observations on this subject that they need not be discussed further.

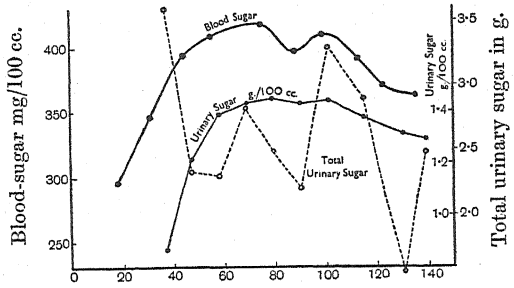
It will be seen that none of the methods above described attains to any high degree of accuracy, and that they are all equally inapplicable in the case of a patient who is never sugar-free. The second method described probably

gives the least distorted results of all, but its limited accuracy has always left doubts as to the validity of conclusions based upon its results.

New method.

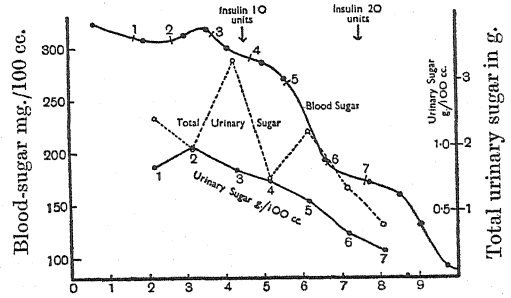
The theoretical basis.

The question of the renal threshold is so intimately bound up with the problem of the relationship of glycosuria to glycaemia that they cannot be discussed apart. These various relationships are well brought out by the data obtained from a sugar tolerance curve, in which frequent observations of blood-sugar and urine-sugar values are made at definite intervals, and then charted on the same time scale. It is immediately obvious that, whilst there is no apparent correspondence between the glycaemia and the total amount of urinary sugar, there is a definite parallelism between the concentration of sugar in the blood and the concentration of sugar in the urine (Figs. 1 and 2).



Time in minutes after administration of 50 g. glucose

Fig. 1.



Time in hours

Fig. 2.

This correspondence is so close that it suggests a proportionate relationship between the two variables. If now the figures for the concentration of glucose in the urine are plotted along the abscissa against points on the ordinate denoting the preceding blood-sugar values at those points in time midway between two consecutive urinations, points are obtained lying along a straight line which cuts the ordinate at some point above zero (Figs. 3, 5 and 7). From

such a graph it is possible to read off the blood-sugar values corresponding to any given concentration of urinary sugar.

Now when the blood-sugar is below and until it attains the renal threshold, no sugar appears in the urine. When the threshold is just reached, sugar is still absent from the urine and immediately it is passed a definite concentration of sugar appears. On such a graph, therefore, the blood-sugar value corresponding to zero on the abscissa is the renal threshold value, and this is the point where the straight line cuts the ordinate.

It will be noted on reference to Figs. 5 and 7 that two lines are obtained. One marked "rise" is drawn through the points obtained whilst the blood-sugar curve is rising, and on the other marked "fall" through those taken during the fall of the curve. This point is well brought out when the data from two consecutive tolerance curves are taken. The two different threshold values obtained correspond respectively to the threshold of appearance and the threshold of disappearance. In Fig. 3 the values charted being taken

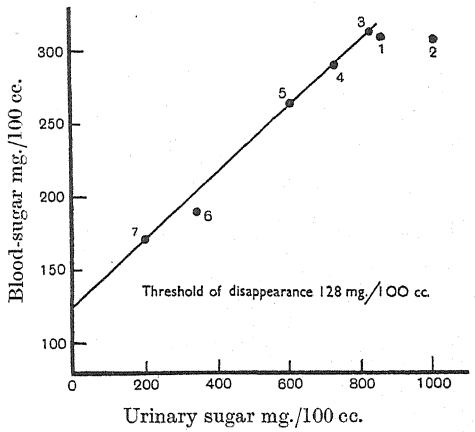


Fig. 3. (Linear graph corresponding to Fig. 2.) Note that the values taken before the fall was established fall off the line.

exclusively from a fall curve (Fig. 2), only the threshold of disappearance is shown. In all cases in which two lines are obtained it will be found that these are never continuous; they are separated by an indeterminate zone corresponding to the higher blood-sugar values found round the peak of the tolerance curve, and in this zone various points interpolated from these values fall. Only in those cases in which the peak is flattened into a plateau can a sufficient number of points be obtained to give some idea of the happenings in this area. From the data shown in Fig. 8 it will be seen (Fig. 9) that these points tend to fall on a smooth curve, in this case continuous with the fall line, which, if continued, would make a sharp angle with the rise line. Consideration of these and other figures suggests that the junction between the two lines is effected, not by a single, but by an S-shaped curve, and that this is not necessarily enclosed in its entirety by the two lines.

From the linear graph it is possible to deduce a formula by which the renal threshold may be obtained. Firstly, it will be seen that for equal variations in blood-sugar values there are equivalent variations in the figures for the concentration of urinary sugar. Secondly, that the ratio between the height of the blood-sugar above the renal threshold and the corresponding urinary sugar is constant.

Let B_1, B_2, B_3 = value of the blood-sugar in mg./100 cc.

Let W_1, W_2, W_3 = value of the urinary sugar in mg./100 cc.

Let x = renal threshold.

Then $B - x$ = head of blood-sugar above the renal threshold.

Now $$\frac{B_1 - x}{W_1} = \frac{B_2 - x}{W_2} = \frac{B_3 - x}{W_3} = K,$$

and when $$B_1 - B_2 = B_2 - B_3,$$

then $$W_1 - W_2 = W_2 - W_3.$$

Consider a blood-sugar value which is $(B_1 - B_2)$ mg./100 cc. above x, *i.e.* $(B_1 - B_2) + x$.

When the blood-sugar falls from B_1 to B_2 the concentration of sugar in the urine falls from W_1 to W_2, *i.e.* $(W_1 - W_2)$ mg./100 cc. Therefore the blood-sugar value $(B_1 - B_2) + x$ corresponds to a urinary sugar concentration $W_1 - W_2$.

Therefore $$\frac{[(B_1 - B_2) + x] - x}{W_1 - W_2} = K = \frac{B_1 - x}{W_1},$$

$$\frac{B_1 - B_2}{W_1 - W_2} = \frac{B_1 - x}{W_1},$$

whence $$x = \frac{W_1 B_2 - W_2 B_1}{W_1 - W_2}.$$

Thus it is theoretically possible to find the renal threshold value from two consecutive figures expressing the urine-sugar in mg./100 cc., and the two corresponding blood-sugar values.

The technique of the method.

The patient is starved for 18 hours before the test is to be performed. During this time he is allowed water *ad lib.*, and at the 14th hour, after emptying the bladder, he is given 1 pint of water. At the 16th hour the bladder is emptied again, another pint of water taken, and at 17½ hours this is repeated. By this means the mucous membrane of the bladder is washed free from any adherent sugar. At the 18th hour the resting blood-sugar is determined, the bladder emptied, and 50 g. of glucose given by mouth. At 10-minute intervals further blood and urine samples are taken, and after each urination the patient is given 300 cc. of water to drink. Each specimen of urine is tested for sugar and only those specimens saved which give a positive reaction.

The collection of the blood offers no difficulty, but various points must be attended to in the collection of the urine. The large amount of water taken

not only removes the discrepancy referred to later, but provides sufficient fluid for the frequent micturitions necessary. Under its influence it will be found quite easy to obtain 10-minute samples from men, but in women it is essential to pass a catheter. The most convenient way of doing this is by means of a male gum elastic catheter complete with a well-fitting spigot. At the beginning of the test this is passed and secured in position by means of tapes and adhesive plaster. All that it is then necessary to do in order to collect a specimen is to remove the spigot and press on the anterior abdominal wall. When the urine ceases to flow the pressure is maintained and the catheter withdrawn slightly to ensure that there is no retention due to the tip projecting too far into the bladder. The point to be insisted on in both sexes is that the bladder be emptied as completely as possible at each urination.

The question now arises as to the efficiency with which the urinary tract can be evacuated and the error introduced into the glucose concentration figures by that degree of urinary retention which is inevitable. Obviously it is impossible to empty the ureter and the pelvis of the kidney, and the urine contained in them will appear in the next specimen. This part of the tract usually contains about 5 cc. of fluid. The diuresis induced during the investigation is of the order of 100 to 200 cc. per 10 minutes. If the blood-sugar values are rising or falling slowly the concentration of urinary sugar rises or falls by small amounts in consecutive specimens, and the correspondence between the two is practically perfect. If the variations are more marked the corresponding error becomes more pronounced, but this can be overcome to some extent by increasing the diuresis. For this reason it is always more important to have a marked diuresis on the rise, which is rapid, than on the fall of the blood-sugar curve, which is slow. The correspondence demonstrated in the ensuing results shows that there is here no serious source of error.

The first specimen, after the renal threshold has been passed on the rise of the curve, will possibly be diluted by the urine passed immediately previous to this. For this reason this specimen may be misleading and in that case, when the figures relative to it are charted, a discrepancy will be revealed. This specimen should not however be discarded, for, as pointed out in the section discussing the accuracy of the method, sugar may have been present in the specimen previous to this but have been diluted beyond the limits of detection of the qualitative reagent, and in that case the first sample giving a positive reaction will contain its full complement of sugar. On the fall a similar discrepancy may arise, for whilst the specimen passed immediately after the fall below the threshold may contain a sufficient concentration of sugar to give a qualitative reaction, this may still fall short of the maximum concentration possible.

We have seen that when the curve is in the region of the peak, values are obtained which on charting give points falling in the indeterminate zone between the two lines. These points are serious sources of error if included in the calculation of the threshold. Therefore, values should always be taken as

near each threshold as possible. Consecutive specimens after the appearance of sugar on the rise fulfil this condition, but in order to obtain similar specimens on the fall one is driven, if the curve is at all prolonged, to forming an opinion, from clinical data, as to the time when the fall will be well established. If the urine passed before the test is commenced contains sugar, it is wise to assume that the curve will take a considerable time to return to the normal level. All the data will be obtained, and the determination considerably shortened, if the blood-sugar is first depressed by the injection of 15 units of insulin and then raised by the subsequent administration of glucose.

It is hardly necessary to point out that if the blood-sugar curve is falling slowly specimens may be collected at longer intervals than 10 minutes, and quite accurate results obtained.

Theoretically, each threshold may be determined from two urine and two blood samples, but in practice it is more accurate to take three specimens of each, to chart them, draw the line which corresponds most closely to the three points, and by reading off two sets of values from this line apply the equation and obtain the threshold.

To obtain the following data MacLean's method was used for the estimation of the blood-sugar, and for the urinary sugar Benedict's method in the early cases and the polarimeter in the later. There have been many criticisms of the estimation of sugar by the polarimeter. The instrument used was a Hilger M 2 with direct vision spectroscope attachment and the light was provided by a mercury vapour lamp. The specific rotation of glucose was taken as $[\alpha]_D + 52{\cdot}5°$ and the readings were obtained for the mercury green line corrected to α_D by dividing with the factor 1·18. The error involved in the reading amounted to 0·02°. With the diuresis obtained the specimens were clear and it was found that such a urine, containing no glucose, showed no rotation.

Lastly it must be emphasised that to obtain good results the conditions laid down above must be adhered to strictly, as under other conditions discrepancies may occur.

The accuracy of the method.

In discussing the accuracy of this method there are two distinct considerations to be taken into account. One is the accuracy of the methods used in the determination of the necessary data, and the second is the accuracy of the method itself relative to these data.

The accuracy attainable by the various methods used in the determination of the blood-sugar is still under dispute and will not be discussed. As regards the urine-sugar, the majority of urines analysed being of low concentration in sugar, Benedict's method did not give an end-point sufficiently sharp, but owing to the conditions of the experiment accurate readings in practically all the specimens containing their full complement of sugar could be obtained on the polarimeter.

With regard to the second consideration, the whole question turns upon the variation in position of the line allowed by the points in the linear graph. The conditions necessary for the obtaining of good results had been laid down when the experiment, set forth in Figs. 4 and 5, was performed. It will be

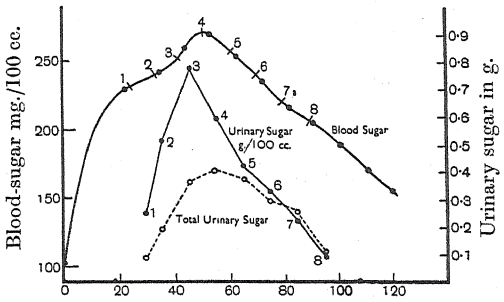


Fig. 4.

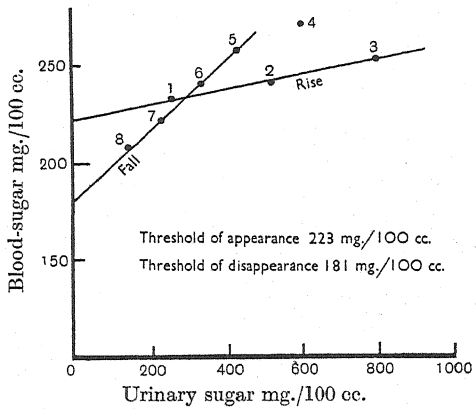


Fig. 5. Linear graph corresponding to Fig. 4.

seen (Fig. 5) that on the fall the line cuts each point and that no variation is possible. The threshold of disappearance is definitely 181 mg./100 cc. On the rise a variation is allowed and the maximum value of this is 2 mg., that is, the threshold of appearance lies between 221 mg./100 cc. and 223 mg./100 cc. Greater variations do occur, but even on the rapidly rising curve of a case of renal glycosuria these should not exceed 6 or 7 mg. of the total blood-sugar, whilst on a slowly moving curve practically no latitude should be allowed.

One of the advantages of the method is that it is not limited by the sensitivity of qualitative reagents. This is well illustrated by case 6 and the case used for Figs. 4 and 5. Consider, in the former, the rising blood-sugar curve. By the above method the threshold of appearance is 250 mg./100 cc. The 250 mark is crossed at 23 mins., but at 28 mins. a urine giving a negative

Benedict's reaction is passed. The preceding specimen, also with a negative Benedict's reaction, was passed at 17 mins. From the blood-sugar curve and linear graph the following data can be adduced.

At 28 mins.	the blood-sugar is	268	mg./100 cc. corresponding to	200	mg./100 cc. urinary sugar.
„ 27	„	266	„	170	„
„ 26	„	262	„	140	„
„ 25	„	258	„	90	„
„ 24	„	254	„	50	„
„ 23	„	250	„	0	„

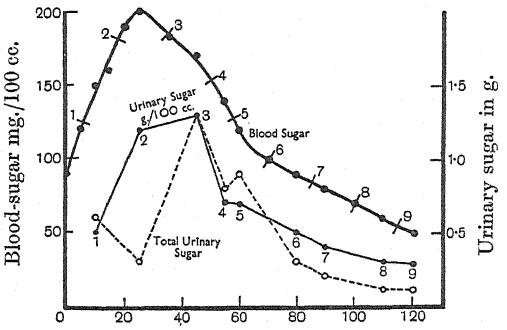


Fig. 6. Case of renal glycosuria. Figures taken from *Acta Med. Scand.* Supp. XXVII, p. 209 (Malmros).

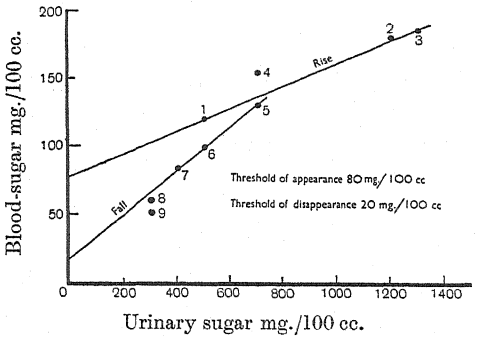


Fig. 7. Linear graph corresponding to Fig. 6.

The average content of sugar in the urine over the 5 mins. is 130 mg./100 cc., but this is diluted by urine passed in the preceding 6 mins., and, if we assume that the diuresis is approximately the same over these 11 mins., then the average concentration of urinary sugar passed at 28 mins. is 60 mg./100 cc., which is undetectable by Benedict's reagent.

In all cases in which the threshold has been determined by the above method, and in which a rough idea of the threshold could be directly obtained, no discrepancy has been found.

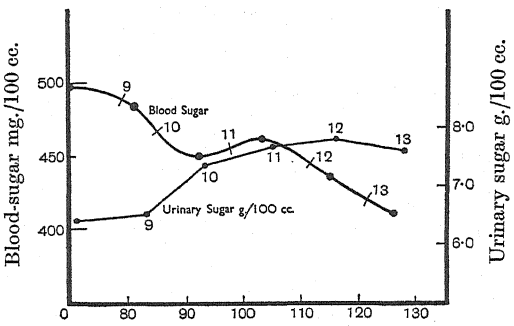
It may then be concluded that even with the limited accuracy of the means of estimation at present available, the method gives practically perfect results.

Results.

In Table I are given the results in 20 cases. Eleven of these have been arrived at from estimations done by the writer, and nine from figures taken from the literature. It is worthy of comment that in few instances, amongst

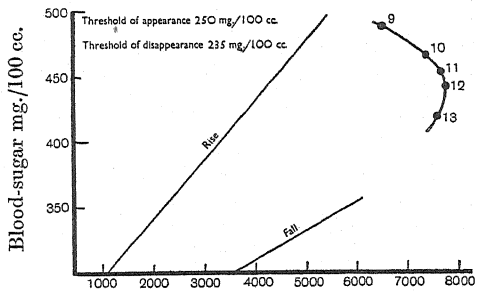
Table I.

Own cases				Cases from literature			
No.	Case	Threshold of appearance mg./100 cc.	Threshold of disappearance mg./100 cc.	No.	Reference	Threshold of appearance mg./100 cc.	Threshold of disappearance mg./100 cc.
1	D.W.	—	128	1	Malmros [1928], p. 209	79	20
2	E.F.	—	232	2	,, p. 241	30	103
3	S.D.	—	266	3	,, p. 210	—	70
4	H.W.	206	200	4	,, p. 233	86	104
5	E.P.	200	195	5	Hatlehol [1924], p. 236	110	142
6	G.L.	250	304	6	,, p. 237	234	224
7	I.H.	160	182	7	Bailey [1919], Case 2	73	50
8	H.P.	250	235	8	,, Case 7	232	192
9	H.M.	223	181	9	Goldblatt and Ellis [1931], Case 3, glucose	129	170
10	M.J.	194	175				
11	A.J.	152	144				



Time in minutes after administration of glucose

Fig. 8.



Urinary sugar mg./100 cc.

Fig. 9. Linear graph corresponding to Fig. 8. The "rise" and "fall" lines are taken from a previous experiment.

the multitude of figures recorded for glucose tolerance curves, were the corresponding percentages of urinary sugar noted, and in some cases in which they were noted the experiments were unsuitable for calculation by reason of their complicated nature. Perfect results are not to be expected from such figures, as the exact time relationships between the urine and blood samples are not recorded.

It will be noted that in no example are the two thresholds at the same level, although in many instances they lie very close together. This latter

Table II.

	Blood		Urine			
Case	Time in mins.	Sugar mg./100 cc.	Time in mins.	Volume cc.	Sugar %	Total sugar g.
6 Diabetic	0	189	—	—	—	—
	19	236	—	—	—	—
	32	282	28	62	0	0
	49	332	47	78	0·538	0·429
	59	350	57	51	0·870	0·444
	75	374	70	78	1·144	0·891
	88	374	85	117	1·238	1·44
	179	349	175	650	0·850	5·52
	190	337	187	120	0·650	0·78
	202	318	199	76	0·385	0·293
	218	308	215	67	0	0
	228	299	—	—	—	—
10 Diabetic	0	—	0	—	0	0
	12	182	14	—	0	0
	24	200	27	—	0	0
	35	216	39	100	0·105	0·105
	46	237	50	83	0·298	0·248
	57	268	60	73	0·52	0·285
	68	288	71	84	0·73	0·6
	78	310	81	82	0·86	0·71
	87	322	90	82	0·99	0·81
	98	330	100	91	1·06	0·96
	108	306	112	120	0·96	1·15
	119	278	121	115	0·72	0·83
	131	253	133	120	0·56	0·67
	142	243	144	112	0·46	0·51
9 Diabetic	0	103	7	—	0	0
	12	208	18	65	0	0
	22	231	29	35	0·250	0·087
	34	244	35	36	0·515	0·196
	43	260	45	47	0·780	0·364
	52	271	54	63	0·598	0·405
	62	254	65	88	0·427	0·375
	72	237	74	90	0·330	0·298
	82	218	85	113	0·226	0·256
	91	207	95	109	0·097	0·105
	101	191	106	93	0	0
	111	172	115	70	0	0
	120	157	—	—	—	—
11 Renal glycosuriac	0	103	—	—	—	—
	14	140	10	24	0	0
	22	177	27	15	0·12	0·028
	40	189	35	14	0·525	0·077
	53	176	49	43	0·25	0·108
	66	160	62	86	0·13	0·111
	82	142	78	170	0·065	0·110
	99	125	—	—	0	0
	119	114	—	—	—	—

apparent correspondence is deceptive, for in every case the two lines giving the thresholds showed distinctly different inclinations.

In Table II the figures from four of the author's experiments are set forth. It will be noticed that the diuresis is not marked in any of them, and we may say at once, that the minimum diuresis required for correspondence varies within wide limits for different individuals.

Discussion.

The primary observation upon which the preceding work is based is that, whilst, under the above conditions, there is a relationship between the concentration of sugar in the urine and the concentration of sugar in the blood, the total amount of sugar in the urine bears no relation to the glycaemia but rather reflects the water output. The important corollary of this observation is that the concentration of sugar in the urine is independent of the diuresis. Now the sugar in the urine is derived from the sugar in the blood, and yet it is impossible to lower the blood-sugar level by creating a diuresis and transferring large amounts of sugar from the blood to the urine. The concentration of sugar in the blood remains steady, the dependent concentration of urinary sugar is unaltered, the total amount of sugar in the urine varies within wide limits. Point is lent to this statement when it is remembered that whilst a patient with a blood-sugar of 300 mg./100 cc. may by diuresis be made to lose 10, 15 or 20 g. of sugar each hour, yet the total amount of sugar in his blood lies between 12 to 15 g. There can only be one conclusion from this phenomenon, and that is that as the body must be supplying sugar to the blood to keep the concentration constant, then the blood-sugar is being maintained at that concentration in spite of all influences to the contrary. Regarded from this point of view the sugar tolerance curve takes on an importance greater than that of a passive expression of the quantity of sugar in the blood awaiting storage, and the high blood-sugar of the diabetic acquires a new significance. Each level of glycaemia must be looked upon as a dynamic rather than a static condition; each as the optimum level for the needs of the body under the particular combination of circumstances then obtaining.

In explanation of the above observations, and the relationships deduced from them, the following theory is offered.

It is a well-known fact that insulin can be demonstrated in the body in tissues other than the pancreas, and that, in a diabetic who has died in coma, insulin may be recovered in surprising quantities from other structures than that organ. It is assumed that in life an insulin factor (I.F.) is present in all living tissues. Under a particular set of circumstances the body requires a definite amount of energy per unit of time, and of this energy each tissue requires its own particular quota. To produce this energy per unit of time a certain amount of carbohydrate must be utilised at a certain rate. Let it be assumed that the utilisation of glucose in the organism for the production of energy involves two consecutive reactions, (*a*) the formation, by the inter-

action of the glucose and tissue I.F., of an insulin-glucose compound, and (*b*) the breakdown of this compound with the simultaneous oxidation of the glucose and the production of energy. It is suggested that these reactions conform with the law of mass action and that the concentration of available oxygen required for reaction (*b*) is constant. In that case the initial velocity of reaction (*b*) will be proportional to the concentration of the insulin-glucose compound which itself is being formed, according to reaction (*a*), at a rate proportional to the product of the concentrations of insulin and glucose. Now under resting conditions the demand of the body for production of energy may be regarded as practically constant. If this demand is satisfied by the degradation of the insulin-glucose compound, the concentration in the tissues of the latter must also remain constant. It follows then that the formation of the insulin-glucose compound is proceeding with unvarying velocity, and therefore that the product of the concentrations of I.F. and glucose, which determine this velocity, is a constant quantity. Thus, when the production of energy is constant, if the concentration of insulin be increased, then a fall in the concentration of the blood-sugar is to be anticipated and *vice versa*.

The common observations of (1) the reduction in blood-sugar concentration following injection of insulin, and (2) the increasing blood-sugar concentration which accompanies the progressive restriction of insulin by disease, would follow as immediate corollaries of the above hypothesis. As a further example on this basis may be explained the results which follow administration of glucose.

It is well known that after the oral administration of glucose to a patient at rest there is a rise and fall in blood-sugar values, immediately preceded by a sharp rise and sharp fall in the respiratory quotient and the metabolic rate. The theory outlined above affords an explanation of these phenomena if the additional assumption be made that an increase in concentration of the blood-sugar does not cause an immediate decrease in that of the I.F. In that case the increase in concentration of the blood-sugar, subsequent to ingestion of glucose, will increase the velocity of reaction (*a*), and the resulting higher concentration of the insulin-glucose compound will bring in its train an increased rate of degradation and hence of energy production. The latter will manifest itself in a raised metabolic rate, the increased degradation in an approximation of the respiratory quotient to unity.

The limit of increase in these related phenomena will be determined by the concentration available in the tissues of I.F. and as this falls the respiratory quotient and metabolic rate will revert to resting values in spite of the persistence of a high blood-sugar. Since the product of the concentrations of insulin and glucose must be maintained at a certain level in order to satisfy the minimal energy requirements of the body, the concentration of the blood-sugar will not be permitted to decrease until the concentration of the I.F. again rises towards normal levels, that is, the delay in return of the

blood-sugar to its normal value will be inversely proportional to the rate of renewal of the I.F. in the tissues. The prolongation of the blood-sugar curve of a diabetic patient whose capacity to produce insulin is deficient receives therefore a logical explanation.

It might be supposed at first sight that the theory proposed above would demand also an increase in metabolic rate as the result of increasing the concentration of insulin-factor by injection of insulin. The absence of an effect of this kind [Himsworth, unpublished] may be ascribed to the extreme readiness with which the relative excess of glucose is removed from the blood and synthesised into glycogen as the result of the inherent, but varying, capacity of most tissues to effect this object, and not attributed to the existence of a mechanism for the reduction of the effective concentration of the insulin-factor by its storage as an inert substance.

We may regard, then, the rise of blood-sugar in the diabetic as comparable with the rise of blood pressure in chronic interstitial nephritis, and both as compensatory phenomena. The examples need not be multiplied, but the theory offers reasonable explanations of phenomena so far removed as the specific dynamic action of carbohydrates, the results obtained in the combined pancreatectomies and hepatectomies of Mann and Magath, and the constancy of the blood-sugar in the face of diuresis.

The secondary problems arising out of the above primary observations centre around the significance of the linear relationship between the concentrations of sugar in the blood and in the urine. The line itself is an expression of the constant ratio which exists between the height of the blood-sugar above the renal threshold and the concentration of sugar in the urine. There is no similar relationship between the latter and the total blood-sugar.

If we consider the relationship in the light of the secretion theory of renal function, then the renal threshold becomes that level of the blood-sugar required to induce in the tubules the secretion of glucose, and from a consideration of the ratio it would appear that secretion proceeds until an equilibrium is established between the concentration of glucose in the lumen fluid and the concentration of the excess of blood-sugar over the renal threshold.

From the point of view of the filtration-reabsorption theory one may say that the failure of reabsorption is proportional to the excess of blood-sugar over the renal threshold. Ni and Rehberg [1930] have shown that the amount of glucose reabsorbed does not rise until a certain level of blood-sugar is reached and then remain constant, but that reabsorption of glucose goes on even when the blood-sugar is far above the normal. It will be noticed that Ni and Rehberg are speaking in terms of amounts, but if their figures are examined some correspondence will be noticed between the concentrations reabsorbed and the blood-sugar values. It appears quite possible then to interpret their results as indicating that the concentration of sugar reabsorbed by the tubules is governed by the blood-sugar. Above the threshold our own

results support this possibility. We know that the urinary sugar is derived from the blood-sugar and because its concentration is proportional to the excess of the latter above the threshold, it is logical to suppose that any removal of glucose from the filtered fluid in its passage through the tubules occurs also in a concentration proportional to the head of blood-sugar above the threshold. The ratio of excess of blood-sugar to urinary sugar concentration is constant above the threshold, and, if the above argument is correct, then the ratio of excess of blood-sugar over the threshold to concentration of reabsorbed fluid is also constant, and as sugar is escaping and appearing in the urine this can only mean that above the threshold the maximum "reabsorption ratio" has been brought into play. Now the excess of sugar is but a fraction of the whole blood-sugar, and it is unreasonable to imagine that the effect of this excess upon the lumen fluid is different from that exercised by the remainder. Thus it would appear that below the threshold the blood-sugar still governs the concentration of the glucose reabsorbed from the glomerular filtrate. The expression $\frac{B-x}{W} = K$ is mathematically untenable in this latter situation and certainly, if the tubules are working at the maximum reabsorption ratio, they are absorbing sugar at a greater concentration than they need to avoid glycosuria. Theoretically this would leave an excess of water in the tubules and if one considers that many volumes of blood must be filtered to provide one volume of urine then the excess would be considerable, and one would expect a diuresis occurring before the threshold was passed to diminish progressively as sugar appeared. But, in experiments in which the intake of water per unit of time is kept constant, this is the reverse of experience. Apparently then the maximum reabsorption ratio does not come into play until the renal threshold is reached, and in that case the renal threshold may be defined as that value of the blood-sugar required to induce the maximum ratio of reabsorption between the concentration of the blood-sugar and the concentration of sugar in the reabsorbed fluid.

The greater the change in concentration of urinary sugar per unit change in concentration of the blood-sugar the more nearly horizontal the line in the graph, and the smaller the relative change the more nearly vertical the line. The inclination of the line or the value of the ratio might thus be regarded as an indication of the permeability of the kidney to glucose. This does not appear to be characteristic of any group of cases and on the basis of it no differentiation of glycosuria into types can be made. The ratio, however, is constant either on the rise or on the fall in any particular experiment, and as it determines the renal threshold, it establishes conclusively the existence of two separate thresholds, a threshold of appearance and a threshold of disappearance. On the mechanism of their differentiation, however, it throws no light.

Reference must now be made to an interesting discrepancy which occurred during the course of the work.

An investigation was carried out in the usual manner, save that no water was given to the patient after the administration of the glucose solution. The

results are charted in Figs. 10 and 11. The blood- and urinary-sugar curves taken on the rise do not run parallel (Fig. 10) and it will be seen that sugar was

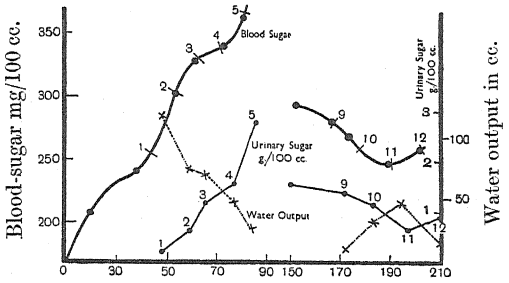


Fig. 10.

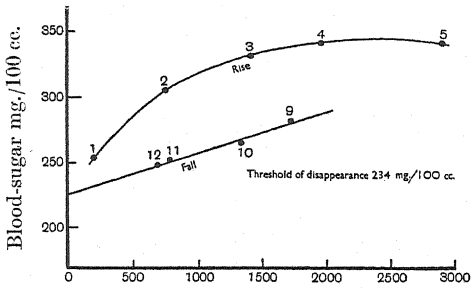


Fig. 11. Linear graph corresponding to Fig. 10.

excreted in concentrations which increased in unexpected proportion to the blood-sugar. After the first specimen the diuresis fell off markedly. In Fig. 11 are shown the lines obtained on charting the two sets of values. The figures obtained on the rise fall on a curve whilst those obtained on the fall lie on the usual straight line. In all the experiments previous to this, determinations had been made on the fall, no attention paid to diuresis, and perfect results obtained. Also in figures taken from the literature with slowly rising blood-sugar curves no discrepancy had been observed. It appears then that with a rapidly rising blood-sugar curve, to obtain correspondence with the urinary sugar, a diuresis is essential.

The interpretation of this observation appears to be as follows. Fluid is filtered off at the glomerulus at a definite level of the blood-sugar, and if owing to diuresis it passes rapidly along the lumen, then when it reaches the tubules it is subjected to the influence of blood of approximately the same concentration as its own precursor and is concentrated proportionately. If, however, it passes slowly down the tubules, whilst the sugar content of the blood is rising rapidly, then it is exposed in the tubules to a blood-sugar value much higher than that at which it was filtered and is concentrated excessively. The result will be a failure of proportionality between urine and blood-sugar figures, and with a progressive fall in diuresis there will be a progressive rise

in the disproportion. On the fall the same argument would explain the occurrence of a more dilute urine than was expected in association with a given blood-sugar, but the slower decrease of the blood-sugar on the fall does not predispose to widely varying sugar contents in the glomerular and peritubule bloods, and thus the discrepancy will tend not to occur.

Poulsson [1930] has shown that if the tubules are paralysed by phlorhidzin sugar behaves as a non-threshold substance, that is, it is excreted *in toto* and the whole mechanism of proportionate excretion previously described presumably breaks down. Thus the tubules appear to be the seat of the proportionate concentration mechanism and in the light of the above experiment it appears justifiable to conclude that the controlling factor of this mechanism is the sugar content of the blood in the peritubule capillaries.

Summary.

1. A review of the literature and methods previously used in the determination of the renal threshold for glucose is given.

2. A new method is described for determining the renal threshold accurately.

3. The results of calculations made from figures taken from the literature and from those obtained in the present investigation are recorded.

4. It is demonstrated that the concentration of urinary sugar is independent of the diuresis, and that it is proportional to the excess of the blood-sugar concentration over the renal threshold.

5. The nature of the renal threshold is discussed in the light of these results and a theory is advanced to explain the lack of dependence of the urinary sugar concentration on the diuresis.

6. Evidence is produced that the concentration of the sugar reabsorbed is determined by the sugar content of the blood in the peritubule capillaries.

In conclusion I should like to thank Sister Freeman for the great assistance she has given me in obtaining urine specimens from female patients, to express my appreciation to Prof. T. R. Elliott for permission to investigate his patients, and to record my gratitude to Prof. C. R. Harington for his help and criticism.

REFERENCES.

Bailey (1919). *Arch. Int. Med.* **23**. 461.
Bernard (1877). Leçons sur le diabète et la glycogénèse animale. (Paris.)
Benedict, Osterberg and Neuwirth (1918). *J. Biol. Chem.* **34**, 217.
Chabanier, Lebert and Lobo-Onell (1929). Physiopathologie et traitement du diabète sucre. (Paris.)
Faber and Hansen (1923). *Acta Med. Scand.* **58**, 372.
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 213.
Frank (1913). *Arch. exp. Path. Pharm.* **72**, 387.
Goldblatt and Ellis (1931). *Biochem. J.* **25**, 221.
Hatlehol (1924). *Acta Med. Scand.* Supp. VIII.
Malmros (1928). *Acta Med. Scand.* Supp. XXVII.
von Mering (1888). *Z. klin. Med.* **15**, 405.
Ni and Rehberg (1930). *Biochem. J.* **24**, 1039.
Poulsson (1930). *J. Physiol.* **69**, 411.
Richards (1929). Methods and results of direct investigation of the function of the kidney. (Baltimore.)
Williams and Humphreys (1919). *Arch. Int. Med.* **23**, 546.

CXXIX. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

III. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF LIVER ESTERASE.

By EDGAR STEDMAN and ELLEN STEDMAN.

From the Department of Medical Chemistry, University of Edinburgh.

(*Received June 27th, 1931.*)

Parts I and II [Stedman, 1926; 1929, 1] of this series of communications have dealt with a new relationship between chemical constitution and physiological action. It has been shown that certain synthetic urethanes possess in common with the alkaloid physostigmine, which is also a urethane, the power of producing a constriction of the pupil when instilled into the eye. As a result of this work, as well as of extensions thereof published elsewhere [Stedman and Stedman, 1929; 1931], it has been concluded that substances which can be classed as phenyl esters of carbamic acids and which possess a basic group will, in general, exhibit similar miotic properties. Constitutional factors which repress, or tend to repress, such properties have already been discussed. Until recently the pharmacological examination of the synthetic urethanes had not extended beyond their action on the eye and it was not therefore certain that they would resemble physostigmine in other physiological properties. One of the urethanes, namely, the methylurethane of *α-m*-hydroxyphenylethyldimethylamine, which had already been shown to possess marked miotic properties and had been named miotine [Stedman, 1929, 2] on that account, has, however, now been submitted to a complete, and three others to an extensive although less complete, pharmacological examination [White and Stedman, 1931]. Miotine has thus been shown to possess physiological properties which are qualitatively identical with those of physostigmine, and similar results have been obtained with the other urethanes as far as they have been examined. The above relationship between chemical constitution and physiological action is therefore not limited to miotic activity but embraces all the known physiological actions of physostigmine. Now, Loewi and Navratil [1926] have attributed the action of physostigmine on the heart to its power of inhibiting the destruction of acetylcholine by an agent which they have shown to be present in the heart and in aqueous extracts of this organ. A similar agent is present in the blood of certain species [Galehr and

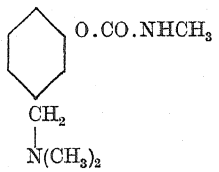
Plattner, 1927]. Loewi and Navratil consider this agent to be an esterase, a view which has been disputed by Plattner and Galehr [1928]. The arguments of Plattner have, however, been disposed of in a recent paper by Engelhart and Loewi [1930], who have brought forward convincing evidence of the enzymic nature of the substance causing the destruction of acetylcholine. Evidence leading to the same conclusion has also been published by Matthes [1930]. In view of the similarity between the actions of miotine and physostigmine on the heart, and in particular of the fact that miotine, like physostigmine, sensitises the vagus and potentiates the action of acetylcholine, it might be expected that miotine would also inhibit the destruction of acetylcholine by the agent in question. That this is actually the case has been shown by Dr Matthes, who kindly compared the action of miotine with that of physostigmine during his investigation of the inhibition by the latter of the destruction of acetylcholine by serum. The problem thus arises as to whether urethanes of the type discussed above, which have been shown to possess similar physiological properties, also share the property of inhibiting the action of the serum-enzyme. Another problem is, however, involved. In the investigations both of Loewi and his co-workers and of Matthes, the destruction of acetylcholine by the solutions under examination has been measured by biological methods, and no experiments appear to be recorded which indicate whether the enzyme which is responsible for this destruction will hydrolyse simple esters, or whether its action is specific towards acetylcholine. If the enzyme is a true esterase it should be capable of hydrolysing simple esters, in which case its activity, as well as the inhibition thereof by drugs, could be followed by titration methods. It appeared to us that these problems could be most conveniently, although indirectly, solved by examining the influence of the urethanes on the activity of the enzyme present in the liver and which is known to hydrolyse simple esters more readily than glycerides. The results of such an investigation are recorded in the present communication.

Materials.

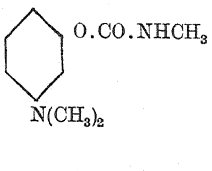
Esterase preparations. All the esterase solutions employed in these experiments have been prepared from pigs' livers by the method of Willstätter and Memmen [1924, 2]. The liver, obtained direct from the slaughter house, was finely minced, desiccated by successive treatment with acetone and ether, and the dry preparation powdered and sieved. Solutions of the enzyme were prepared, as required, by extracting 2 g. of this fine powder with 100 cc. of 0·025 *N* ammonia for 1 hour and removing the insoluble material in the centrifuge. In the earlier experiments no further purification of the esterase was attempted. In the later work, however, it was found to be advantageous carefully to acidify the ammoniacal extract with acetic acid, and, after removing the precipitate so produced, to dialyse the clear solution in collodion membranes for 3–4 days, a procedure similar to that first described by

Willstätter, Bamann and Waldschmidt-Graser [1928] and subsequently employed by other workers.

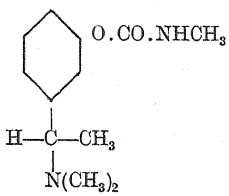
Substances examined for inhibitory action. The urethanes with a pharmacological action similar to that of physostigmine which have been examined for inhibitory action comprise three series of isomeric compounds, namely, the methylurethanes of *o*-, *m*- and *p*-hydroxybenzyldimethylamine, the *o*-, *m*- and *p*-dimethylaminophenyl esters of methylcarbamic acid, and the methylurethanes of the isomeric *α*-hydroxyphenylethyldimethylamines. The structures of the *m*-isomerides in these three series are represented in formulae I, II and III respectively. These compounds have been examined both in the



I



II



III

form of their hydrochlorides and methiodides, the preparation of which has been effected by the methods of Stedman [1926; 1929, 1] and Stedman and Stedman [1929]. In the case of the *o*-isomerides, the hydrochlorides of which are hygroscopic, the base was dissolved in the calculated quantity of hydrochloric acid immediately before use. It should be noted that the urethane represented by formula III is the substance which has been named miotine. As will be evident from the formula, miotine and its position isomerides contain an asymmetric carbon atom. Unfortunately attempts to resolve these compounds have not so far met with success; the examination of their inhibitory actions towards liver esterase has therefore necessarily been carried out with the racemic substances. It will be convenient in the following pages to refer to the isomeric compounds corresponding with formula III as *o*-, *m*- and *p*-miotine, and to those corresponding with formulae I and II as the methylurethanes of the benzyl and phenyl series respectively.

In addition to urethanes with a physostigmine-like action, the methylurethane of choline iodide, $CH_3NH.CO.OC_2H_4N(CH_3)_3I$ [Stedman, 1929, 1], has been examined as an example of an aliphatic urethane. Some tests have also been made with the following alkaloids: pilocarpine, arecoline, ricinine. The two former were employed as nitrate and hydrobromide respectively, these salts being obtained from commercial sources. The ricinine was prepared from castor-oil seeds; since this alkaloid is soluble in water it was employed in the form of the free base.

Pilocarpine and arecoline were examined because the pharmacological actions of these alkaloids are similar to that of physostigmine. Ricinine does not exhibit such similarities. It occurs, however, in the castor-oil seed associated with a lipase, and since physostigmine also occurs in a seed, the Calabar

bean, it was thought that if the two alkaloids were found to resemble one another with respect to their inhibition of the activities of enzymes, this might form the basis of an explanation of the functions of alkaloids in general. As will be shown below, ricinine does not inhibit the activity of liver esterase; its possible influence on the activity of ricinus lipase, the enzyme with which it is associated naturally, has not yet been investigated.

Experiments with methyl butyrate as substrate.

Technique. The activity of the esterase preparations has been determined by a method essentially similar to that developed by Willstätter and Memmen [1924, 1] for pancreatic lipase and subsequently adapted by the same authors [1924, 2] for use with liver esterase. For convenience of measurement, 1 cc. of substrate (methyl butyrate) was used for each 100 cc. of reaction mixture, as recommended by Bamann [1929]. The general procedure was as follows: 20 cc. of ammonia-ammonium chloride buffer (1 part 2·5 *N* NH_3 : 2 parts 2·5 *N* NH_4Cl; p_H 8·9) contained in a 100 cc. measuring flask were diluted with 65–70 cc. of water, the exact volume depending on the volume of enzyme solution subsequently added, and the mixture brought to 30° in a thermostat. 1 cc. of methyl butyrate was then added and the flask shaken vigorously until the ester had dissolved completely. To the clear solution a measured volume (5 or 10 cc.) of the enzyme preparation was added, the total volume of the mixture brought to 100 cc. by the addition of water, and the whole thoroughly mixed. 20 cc. of the mixture were immediately withdrawn, run into a mixture of 25 cc. of 0·2 *N* hydrochloric acid with 20 cc. of water, and titrated with approximately 0·2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar titrations were made at intervals of 20 minutes. With this procedure and using a suitable quantity of enzyme the amount of hydrolysis was approximately proportional to the time.

The enzyme solution consisted, in the earlier experiments, when ammoniacal extracts of liver powder were employed, of 5 cc. of a mixture of 5 cc. of the extract with 1 cc. of water, the latter being replaced by 1 cc. of a solution of the substance under examination in the experiments designed to test the inhibitory action of this substance. In the later experiments, in which the acidified and dialysed esterase preparation was employed, 5 cc. of enzyme solution were mixed with 5 cc. of ammonia-ammonium chloride buffer (of above composition but diluted 1 in 10), 2 cc. of water or solution added, and 10 cc. of this mixture used for the hydrolysis.

Activity of esterase preparations. The activity of the esterase preparations made from different livers was fairly constant. The following figures, which represent the number of cc. of 0·194 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively, using 4·17 cc. of an ammoniacal extract of liver powder in 100 cc. of reaction mixture, may be taken as typical: 2·3, 4·75, 7·25. This result was

obtained on May 19th, 1930, with a fresh extract of a liver powder (L.P. 2) prepared on April 23rd, 1930, and almost identical values were obtained on June 10th, 1930, with a similar extract from the same powder. These facts are recorded in detail because of the following remarkable phenomenon which was observed with an extract of this powder but which has not so far been encountered with other preparations. On June 23rd, 1930, a fresh ammoniacal extract of L.P. 2 was rendered just acid to litmus by the addition of 0·5 *N* acetic acid. The precipitate, which formed slowly, was centrifuged off and the clear solution tested for activity under precisely the same conditions as those employed for the above ammoniacal extract, when the following figures were obtained: 4·45, 8·25, 8·95. Owing to the unexpectedly high activity of this extract the hydrolysis of the substrate was virtually complete in the experimental period of 1 hour, with the result that the reaction did not take the usual linear course. It is therefore not possible accurately to compare the activities of the different extracts of L.P. 2, but it is clear from the figures quoted that the activity of the acidified extract was at least twice as great as that of the ammoniacal extracts previously prepared from the same weight of the same powder. Dialysis of the acidified extract for 4 days was accompanied by an apparent slight diminution in activity, as is shown by the following titration figures: 3·4, 6·7, 8·35; this, however, was mainly due to the dilution which occurred during dialysis. The increase in activity observed on acidification can doubtless be explained by the removal of an inhibitory substance; from the difficulty in reproducing this experiment it appears that a very critical adjustment of conditions, probably of p_H, is necessary for the precipitation of this inhibitor.

Influence of time of contact between inhibitor and enzyme on inhibitory action. Rona and Bach [1920], in their studies on the inhibitory action of atoxyl on various lipases, have shown that the maximum inhibitory effect of this substance is not developed unless it is left for a certain period of time in contact with the enzyme before the addition of the substrate. A number of preliminary experiments having demonstrated that urethanes of the miotine type exert a pronounced inhibitory action on the hydrolysis of methyl butyrate by liver esterase, the influence of this time of contact of the inhibitor with the enzyme was investigated. For this purpose, 1 cc. of a solution of miotine hydrochloride, containing 1·2 mg./cc., was added to each of 7 test-tubes containing 5 cc. of an ammoniacal extract of liver powder. After varying intervals of time 5 cc. of the mixture were employed for a hydrolysis experiment. The results are given in Table I. The figures indicate that, under the conditions employed, the maximum inhibitory action of miotine hydrochloride is not exerted unless it has been in contact with the esterase for 2 hours prior to the addition of the substrate. The difference between the maximum inhibitory action and that produced after contact for 1 hour is, however, very small and almost falls within the limits of experimental error. It has therefore been considered sufficient, when comparing the inhibitory activities of the various

Table I. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*

Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. T = 30°.

Time of contact (mins.)	Titration figures (cc. of 0·194 *N* NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	2·5	5·15	7·75	—
0·1	1·8	3·7	5·6	28
15	0·9	1·95	3·0	61
30	0·75	1·5	2·25	71
45	0·6	1·25	1·9	75
60	0·5	1·1	1·7	78
120	0·4	0·9	1·5	81
330	0·4	0·9	—	81

urethanes, to allow the inhibitor to stand in contact with the enzyme for 1 hour before use.

The figures in Table I give some further important information. Miotine is an ester of methylcarbamic acid and might therefore be expected to suffer, like other esters, a more or less rapid hydrolysis when in contact with liver esterase. In the above experiments, however, it is clear that no appreciable hydrolysis has occurred in a period of $5\frac{1}{2}$ hours. In this connection it should be mentioned that, although the hydrolysis experiments were carried out at 30°, the mixtures of miotine and esterase were allowed to stand at room temperature before addition to the reaction flask.

Some experiments on the influence of the time of contact of enzyme and inhibitor on the inhibitory action of the latter were also made under somewhat different conditions. Although the procedure employed in these experiments was not adopted in much of the subsequent work, the results are recorded here since they are not entirely without interest. In the preceding experiments the enzyme and inhibitor were in contact under conditions of concentration widely different from those obtaining in the final reaction mixture. In order to render these conditions more nearly comparable the following modified series of experiments was carried out. A solution of 1·2 mg. of miotine hydrochloride in 1 cc. of water was added to each of a series of flasks containing the diluted buffer mixture and enzyme (ammoniacal extract) previously warmed to 30°. After varying intervals of time, 1 cc. of methyl butyrate was added and rapidly dissolved by shaking, the volume adjusted to 100 cc., and the titrations were performed as before. The results are expressed in Table II. Unfortunately a quantitative comparison cannot be made between the results of Tables I and II since not only were different enzyme preparations employed but the amounts of inhibitor used were slightly different. Nevertheless it is evident that the inhibitory action of miotine hydrochloride is of the same order of magnitude in the two sets of experiments. The most striking point of difference is the definite indication of a destruction of the miotine in the second set after 4 hours' contact with the enzyme. We are inclined, however, to attribute this destruction to the alkalinity of the

Table II. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*

Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. T=30°.

Time of contact (mins.)	Titration figures (cc. of 0·194 *N* NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	1·95	4·00	6·1	—
15	0·7	1·5	2·35	61
30	0·6	1·25	1·7	72
45	0·4	1·0	1·4	77
60	0·4	0·85	1·3	79
120	0·45	1·0	1·6	74
240	0·8	1·65	2·45	60

solution, combined with the fact that the enzyme and inhibitor were maintained at 30° during the whole period of contact, rather than to the action of the esterase.

Inhibitory actions of various urethanes. A number of the urethanes exhibiting miotic activity were examined for inhibitory action under fairly wide ranges of concentration of inhibitor. The results are collected in Table III.

Table III. *Inhibitory action of various urethanes with miotic activity.*

Substrate: methyl butyrate. T=30°. Initial p_H=8·9.

In the three instances marked (*a*), 10 cc. of the extract were employed owing to the diminution in the activity of the liver powder.

The solution used in the experiment marked (*b*) was prepared by extracting only 1·5 g. of liver powder with 100 cc. of *N*/40 ammonia.

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
m-Miotine HCl	L.P. 2	Control	2·55	5·15	7·8	—
		10	0·2	0·5	0·9	89
		1	0·45	1·0	1·45	81
		0·1	1·25	2·6	4·05	48
		0·01	2·1	4·3	6·55	16
		0·001	2·4	4·8	7·3	6
o-Miotine HCl	L.P. 5 (*a*)	Control	2·15	4·35	6·5	—
		10	0·55	1·0	1·45	78
		1	1·6	3·3	4·9	25
		0·1	1·8	3·75	5·9	11
p-Miotine HCl	L.P. 2	Control	2·35	4·75	7·1	—
		10	0·0	0·3	0·45	94
		1	0·25	0·55	0·85	88
		0·1	0·9	1·5	2·55	64
		0·01	1·65	3·3	5·0	30
		0·001	2·35	4·5	6·85	4
m-Miotine MeI	L.P. 2	Control	2·45	5·0	7·5	—
		10	0·25	0·5	0·85	90
		1	1·25	2·4	3·75	51
		0·1	2·15	4·4	6·75	11
		0·01	2·15	4·8	7·2	4
		0·001	2·3	4·8	7·3	—
o-Miotine MeI	L.P. 5 (*a*)	Control	2·0	4·2	6·4	—
		10	0·25	0·7	1·1	83
		1	1·2	2·5	3·6	44
		0·1	2·05	4·1	6·2	3

Table III (*contd.*).

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
p-Miotine MeI	L.P. 5 (*b*)	Control	1·75	3·4	5·2	—
		10	0·15	0·35	0·65	86
		1	0·85	1·55	2·35	55
		0·1	1·4	3·0	4·5	14
		0·01	1·6	3·25	5·0	—
		0·001	1·45	3·3	5·0	—
HCl of *o*-urethane (phenyl series)	L.P. 6	Control	2·5	5·1	7·7	—
		10	0·2	0·6	1·0	87
		1	0·6	1·2	1·85	77
		0·1	1·5	3·05	4·75	38
		0·01	2·05	4·15	6·4	17
		0·001	2·2	4·55	7·05	8
HCl of *m*-urethane (phenyl series)	L.P. 2	Control	2·45	4·85	7·5	—
		10	0·0	0·1	0·3	96
		1	0·35	0·5	0·75	90
		0·1	1·15	2·1	3·25	57
		0·01	1·8	3·55	5·45	27
		0·001	2·1	4·6	6·8	9
HCl of *p*-urethane (phenyl series)	L.P. 2	Control	2·3	4·75	7·4	—
		10	0·25	0·5	0·85	89
		1	0·7	1·35	2·0	73
		0·1	1·7	2·25	3·95	33
		0·01	2·2	4·4	6·65	11
		0·001	2·2	4·55	7·0	6
MeI of *m*-urethane (phenyl series)	L.P. 2	Control	2·45	5·1	7·6	—
		10	0·75	1·5	2·4	69
		1	1·7	2·35	3·95	37
		0·1	2·15	4·55	6·9	11
		0·01	2·25	4·75	7·2	5
		0 001	2·35	4·8	7·3	—
MeI of *p*-urethane (phenyl series)	L.P. 5 (*a*)	Control	2·1	4·4	6·55	—
		10	0·6	1·15	1·7	74
		1	1·35	2·65	4·0	39
		0·1	2·2	4·3	6·45	—
MeI of *o*-urethane (benzyl series)	L.P. 2	Control	2·35	4·75	7·3	—
		10	0·2	0·45	0·75	90
		1	1·15	2·3	3·4	53
		0·1	2·3	4·6	7·0	4
		0·01	2·4	4·75	7·25	—

In all these experiments the general technique described earlier was utilised, ammoniacal extracts of desiccated liver powder being employed as esterase solution in each case. Since this work was carried out over an extended period of time, different liver preparations, as indicated in Table III, were necessarily employed owing to the moderately rapid deterioration which such preparations undergo. The results demonstrate clearly the marked inhibitory action which urethanes of the type examined exert upon the activity of liver esterase. A detailed comparison of the figures shows further that, in general, the inhibitory activity of the hydrochlorides of the tertiary bases is greater than that of the corresponding quaternary iodides. While this is not universally the case, particularly in the higher concentrations, the inhibitory activities of the methiodides consistently decrease, with increasing dilution of the inhibitor, at a much greater rate than do those of the hydrochlorides of the tertiary

bases, with the result that at low concentrations the latter are uniformly more active. This difference between the two types of salts is emphasised because it is probably related to the mechanism of the inhibitory action.

Examination of other types of compounds for inhibitory activity. The following compounds were tested for inhibitory activity under conditions similar to those used in the preceding experiments: methylurethane of choline iodide, hydrochloride of *α-m*-hydroxyphenylethyldimethylamine, pilocarpine nitrate, arecoline hydrobromide, atropine hydrochloride and ricinine. The titration figures need not be recorded in detail since in no case was any inhibitory action observed in the highest concentrations examined, namely, 10 mg. per 100 cc. of reaction mixture.

Influence of p_H *on inhibitory action.* The technique adopted for the experiments in which methyl butyrate was employed as substrate is not very suitable for the investigation of p_H effects. Even when relatively large amounts of buffer are employed, the acid liberated during the hydrolysis is sufficient to cause some change in p_H; if the solution is thereby brought to the acid side of neutrality, some loss in the activity of the esterase may occur with the result that the hydrolysis of the substrate will no longer proceed at a constant rate. This effect was actually observed in a number of experiments. In the experiment quoted in Table IV, which was carried out at p_H 6·8, proportionality between the amount of hydrolysis and the time was maintained;

Table IV. *Influence of* p_H *on the inhibitory action of miotine hydrochloride.*

Substrate: methyl butyrate. T = 30°.

p_H	Esterase preparation	Conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
6·8	Acidified ammoniacal	Control	1·65	3·4	5·0	—
(phosphate buffer)	extract of L.P. 5	10	0·5	1·25	2·0	60
		1	1·45	2·9	4·35	13
		0·1	1·6	3·2	4·85	3
		0·01	1·75	3·4	4·95	—
		0·001	1·7	3·5	5·15	—
8·9	Ammoniacal extract	Control	1·85	3·8	5·75	—
(ammonia buffer)	of L.P. 5	12	0·05	0·25	0·45	92
		1·2	0·35	0·75	1·15	80
		0·12	1·0	2·25	3·5	39
		0·012	1·65	3·3	4·9	15
		0·0012	1·85	3·6	5·45	5

this experiment therefore serves to illustrate the general influence of p_H on the inhibitory action of miotine hydrochloride. For comparison, the results of an experiment at p_H 8·9 carried out under similar conditions are included in the same table. The technique described in connection with the experiments quoted in Table II was employed for both experiments. The phosphate buffer used consisted of a mixture of equal parts of $M/3$ Na_2HPO_4 and $M/3$ NaH_2PO_4, 70 cc. being employed in each 100 cc. of reaction mixture. When this buffer was employed, phenolphthalein was used as indicator for the titrations.

Unfortunately the enzyme solutions employed in these experiments, although prepared from the same liver powder, were of different degrees of

purity; the results are not therefore strictly comparable. Nevertheless, two important conclusions can be drawn from these experiments: firstly, the inhibitory action of miotine hydrochloride is smaller on the acid than on the alkaline side of neutrality; secondly, the inhibitory action falls off with decreasing concentration of inhibitor much more rapidly in acid than in alkaline solution.

Comparison of the inhibitory activities of isomeric urethanes. The urethanes which have been shown above to inhibit the hydrolysis of methyl butyrate by liver esterase are members of three series of isomeric compounds, with their corresponding methiodides. The results do not, however, necessarily indicate the relative inhibitory activities of the position isomerides within a given series or even of members of different series, for, as previously mentioned, the inhibitory activities of the various compounds were examined on different esterase preparations, and since the latter were made from different liver powders at varying times after their preparation they possessed different activities and probably contained varying amounts of natural inhibitors. On the assumption that any natural inhibitors which may be present in the enzyme preparation enter into competition with the urethanes, it is clear that in experiments designed to compare the activities of different compounds it is not sufficient to employ the same esterase activity; the comparison can only be regarded as valid in those cases where the same esterase extract has been employed. Some experiments, recorded in Table V, have consequently been carried out in which a comparison has been made of the inhibitory activities of the members of each series of urethanes towards the same enzyme preparation. Finally, the hydrochlorides of the *m*-isomerides of the three series have been compared under the same conditions as a measure of the relative activities of the three series of compounds. In all cases the ammoniacal extracts of the liver powders were purified by acidification with acetic acid followed by dialysis.

From the magnitude of the titration figures it is evident that the percentage inhibition shown in the last column of Table V must be subject to an error of a few per cent. Bearing this in mind, the more significant results revealed by a study of this Table may be summarised as follows.

Miotine series. The inhibitory activities of the hydrochlorides of the *m*- and *p*-isomerides are identical, that of the *o*-isomeride being considerably smaller; the inhibition produced by the latter is, in fact, slightly smaller than that caused by the two former when present in one-tenth of its concentration. No very great difference can be observed between the activities of the methiodides, the *m*- and *p*-isomerides showing about equal activity and the *o*-compound exhibiting a slightly greater effect. It is noteworthy and exceptional that the inhibitory activity of the hydrochloride of the *o*-isomeride is somewhat smaller than that of its methiodide in the same molar concentration.

Phenyl series. The activities of the hydrochlorides are definitely in the order $m > p > o$. The methiodides follow the same order; the *m*- and *p*-compounds show, however, practically identical activities.

Table V. *Comparison of inhibitory activities of various urethanes.*

Substrate: methyl butyrate. T = 30°. Initial p_H = 8·9.

Inhibitor (methylurethane)	Esterase preparation	Final conc. of inhibitor (molar × 10^{-3})	Titration figures			Percentage inhibition
Miotine series:	L.P. 6	Control	1·75	3·5	5·3	—
m-HCl		1/30	0·25	0·65	1·05	80
,,		1/300	1·15	2·4	3·7	30
p-HCl		1/30	0·4	0·8	1·2	77
,,		1/300	1·25	2·45	3·85	27
o-HCl		1/3	0·75	1·55	2·3	57
,,		1/30	1·5	2·95	4·5	15
m-MeI		1/3	0·7	1·35	1·9	63
,,		1/30	1·65	3·2	4·9	8
p-MeI		1/3	0·75	1·15	1·85	65
,,		1/30	1·4	2·9	4·4	17
o-MeI		1/3	0·4	0·8	1·25	77
,,		1/30	1·45	2·9	4·3	19
Phenyl series:	L.P. 6	Control	1·6	3·35	5·1	—
m-HCl		1/300	0·15	0·45	0·7	86
p-HCl		,,	0·8	1·65	2·6	49
o-HCl		,,	1·0	2·05	3·2	37
m-MeI		1/30	0·9	1·75	2·55	52
p-MeI		,,	0·75	1·6	2·55	50
o-MeI		,,	1·3	2·6	4·0	22
Benzyl series:	L.P. 7	Control	2·4	4·75	6·85	—
m-HCl		1/300	0·8	1·75	2·55	63
p-HCl		,,	1·35	2·75	3·95	42
o-HCl		,,	1·85	3·6	5·7	17
m-MeI		1/30	1·45	2·8	4·3	37
p-MeI		,,	0·75	1·5	2·25	67
o-MeI		,,	1·25	2·55	3·85	44
All series:	L.P. 7	Control	2·15	4·55	6·75	—
m-Benzyl-HCl		1/3000	1·95	3·8	5·85	13
m-Phenyl-HCl		,,	1·15	2·45	3·6	47
m-Miotine-HCl		,,	1·5	3·1	4·7	30
	L.P. 7	Control	2·4	4·75	6·85	—
m-Benzyl-HCl		1/300	0·8	1·75	2·55	63
m-Phenyl-HCl		,,	0·1	0·35	0·45	93
m-Miotine-HCl		,,	0·35	0·6	0·8	88

Benzyl series. In this series the order for the hydrochlorides is $m > p > o$, and for the methiodides $p > o > m$.

Comparison of the three series. The inhibitory activities of the hydrochlorides of the *m*-isomerides in equimolar concentrations are definitely different, the order being phenyl > miotine > benzyl.

The results in Table V may be further utilised in order to direct attention to the varying activities of the urethanes with different esterase preparations. Thus, to take the most marked case, the concentrations of miotine hydrochloride required to inhibit the acidified and dialysed extracts of two esterase preparations (L.P. 6 and L.P. 7) to exactly the same extent (30 %) are in the ratio 1:10, the extract from L.P. 6 requiring a final concentration of 1/300,000 *M* and that from L.P. 7 one of 1/3,000,000 *M*. The different responses of these enzyme preparations are doubtless related to their histories, which are as follows: the liver powder L.P. 6 was prepared on January 9th,

1931. An ammoniacal extract of this was made on January 14th and its activity tested by the standard procedure used in this investigation, when the following titration figures were obtained: 2·3, 4·6, 7·0. Practically identical values were obtained on the same day following the acetic acid treatment. The acid solution was then dialysed for 5 days, the dialysed solution giving, on January 19th, titration figures of 2·05, 4·05, 6·05. The diminution in activity shown by these figures is only apparent and can be accounted for by the slight dilution which the extract had undergone during dialysis. On the following day, however, the titration figures for the dialysed extract had fallen to 1·75, 3·5, 5·3. Thus, in 24 hours the activity had diminished about 12 %, and it was this extract with lowered activity which was employed for the comparison of the inhibitory activities of the urethanes of the miotine series. We are of opinion that this loss in activity can be attributed to the acidity caused by the relatively long period of dialysis, and we have found that, with the highly permeable collodion bags which we employ, such losses consistently occur when the process of dialysis is extended beyond 3 or, at most, 4 days.

The preparation L.P. 7 was made on February 18th, 1931. An ammoniacal extract was prepared on February 20th, and this was acidified and dialysed until February 23rd. In view of the fact that this extract, which was more sensitive to miotine hydrochloride than the one referred to above, proved to be stable, it appears probable that the presence of inactivated esterase in the enzyme solutions diminishes the inhibitory activity of urethanes of the type under discussion.

Experiments with tributyrin as substrate.

Technique. In order to follow the hydrolysis of tributyrin by liver esterase the stalagmometric method devised by Rona and Michaelis [1911] and subsequently employed by Willstätter and Memmen [1923] in connection with pancreatic lipase has been utilised. A straight stalagmometer with a water value of 81 at 20° was employed throughout the experiments.

In using this method it is frequently considered sufficient to work at room temperature without any special temperature control. At the time our experiments were carried out, however, the temperature of our laboratory fluctuated considerably and tended to be somewhat low, falling at times to below 10°. At such temperatures the activity of the enzyme is naturally considerably diminished, and it was therefore considered advisable to use a thermostat. The reaction mixture, contained in a small beaker, was placed in a bath at 20° and the stalagmometer supported directly over the beaker by means of a suitable stand. By raising the beaker and applying gentle suction at the top of the stalagmometer the mixture could be drawn into the latter. The beaker was then again lowered and the drops from the stalagmometer allowed to fall into it. By this arrangement fluctuations in the temperature of the liquid actually in the stalagmometer could not be prevented, but the

bulk of the reaction mixture was maintained constantly at 20°, thereby permitting a uniform action of the esterase.

The remaining procedure was as follows: 50 cc. of a saturated solution of tributyrin were mixed with 5 cc. of *M*/3 phosphate buffer (p_H 7·9 or 6·8) and the mixture brought to 20°. 1 cc. of a mixture of 1 cc. of esterase solution with 1 cc. of water in the control experiment, or of 1 cc. of esterase solution with 1 cc. of a solution of the urethane in the experiments designed to measure the inhibitory action of the latter, was then added and the drop number immediately measured. Two further counts were made at intervals of 20 minutes. As in the experiments with methyl butyrate, the enzyme was left in contact with the urethane for 1 hour before mixture with the substrate.

All the esterase solutions employed were prepared from ammoniacal extracts of liver powders acidified with acetic acid; in some cases the extracts were dialysed. In each case, 1 to 5 cc. of the enzyme preparation, the actual volume depending on its activity, were mixed with 2 cc. of *M*/3 phosphate buffer of the same p_H as that employed in the hydrolysis experiments, and diluted to 10 cc. with water.

Willstätter and Memmen [1923] have pointed out that commercial preparations of tributyrin frequently contain an impurity, with a saponification number corresponding with that of dibutyrin, which renders them unsuitable for use, without extensive purification, in the stalagmometric estimation of lipolytic activity. As a criterion of purity they recommend the preparation of two saturated aqueous solutions of the sample, one by shaking 5 to 10 g. of the tributyrin with 200 cc. of water, and the other by shaking 3 drops with a similar volume of solvent. If the tributyrin is pure, the two solutions should give the same drop number. The experiments recorded in this communication have been made with one delivery of B.D.H. tributyrin. When this was submitted to the test described above, the two saturated aqueous solutions gave identical drop numbers. According to the above criterion the sample was therefore quite pure and was employed in our experiments without further treatment.

Influence of p_H *on inhibitory action.* The study of the inhibition of the hydrolysis of tributyrin by liver esterase by urethanes of the type considered in this series of communications has been carried out partly with the object of ascertaining if the inhibitory action of such urethanes is independent of the type of substrate employed and partly because, owing to the sensitivity of the stalagmometric method, only relatively small amounts of tributyrin need be employed as substrate. This renders the method eminently suitable for a study of the influence of p_H on the inhibitory process, since no difficulty is experienced in buffering the small amounts of acid which are liberated by the action of the enzyme. The inhibitory activities of the hydrochlorides and methiodides of each member of the three series of urethanes mentioned above have therefore been examined, using a range of concentrations of inhibitor, at two different acidities, namely, p_H 7·9 and 6·8. Table VI gives some typical

Table VI. *Influence of* p_H *on the inhibitory action of various urethanes.*

Substrate: tributyrin. T=20°.

Inhibitor	Final conc. of inhibitor (mg. per 56 cc.)	p_H 7·9 Decrease in drop number in 20 mins.	p_H 7·9 Decrease in drop number in 40 mins.	p_H 6·8 Decrease in drop number in 20 mins.	p_H 6·8 Decrease in drop number in 40 mins.
m-Miotine HCl	0	14	25	12	20
	10	0	1	1	1
	1	1	1	5	8
	0·1	0	1·5	9	16
	0·01	8	17	10	18
	0·001	13	24	—	—
m-Miotine MeI	0	9·5	19	9	18
	10	0	1	5·5	11
	1	2	4	8	16
	0·1	7·5	15·5	—	—
HCl of *p*-urethane (benzyl series)	0	10	20	11	20
	10	0	0	0	0
	1	0	0·5	0·5	1
	0·1	1·5	3	9	15
	0·01	8	15	9	17
MeI of *p*-urethane (benzyl series)	0	9·5	18·5	8·5	15·5
	10	0	0	0·5	1·5
	1	1	1·5	2	3·5
	0·1	5	10	8	16
HCl of *m*-urethane (phenyl series)	0	9·5	17·5	7	14·5
	1	1	1	1	1·5
	0·1	1	1·5	0	1
	0·01	0	1	1	2
	0·001	1	3	2	4
	0·0001	4	9	7	14
MeI of *m*-urethane (phenyl series)	0	9	19	8·5	15·5
	10	0	0	2	2
	1	0	0	5	9
	0·1	2	4	—	—
	0·01	7	14	—	—

results. Similar results were obtained with the isomerides of the urethanes mentioned in Table VI; since, however, the same enzyme preparation was not employed with all the compounds, these results do not necessarily indicate correctly the relative inhibitory activities of the various compounds and are not therefore quoted. It should be mentioned in connection with these results that the same enzyme preparation was always employed in the experiments with a given urethane, although, owing to the smaller activity of the esterase at p_H 6·8 a greater volume of the enzyme preparation was used in the more acid solution. Thus in the experiments with the hydrochloride of the methylurethane of *p*-hydroxybenzyldimethylamine in which the enzyme showed the same activity, corresponding with a decrease of 20 in the drop number in 40 minutes, at the two acidities, 33 % more enzyme was employed at p_H 6·8 than at 7·9. In every instance quoted in Table VI the inhibitory activity of a given urethane is smaller at p_H 6·8, *i.e.* on the acid side of neutrality, than at p_H 7·9, and the same effect was consistently observed with the remaining urethanes. As an example, the results with the hydrochloride of the methylurethane of *p*-hydroxybenzyldimethylamine, mentioned above, may be taken,

since identical esterase activities, as shown by the control experiments, were employed at the two acidities. In this case, the amounts of inhibitor required to diminish the activity of the enzyme by 25 %, *i.e.* to diminish the decrease in drop number from 20 to 15, were 0·01 and 0·1 mg. at p_H 7·9 and 6·8 respectively. The inhibitory activity of the urethane at p_H 6·8 was thus only one-tenth of that which it exhibited at p_H 7·9. Ratios of the same order of magnitude were obtained with the other urethanes. To what extent the partial inactivation of the enzyme which takes place in acid solution, and which necessitated the employment of larger volumes of the preparation at p_H 6·8, contributes to this result cannot at present be determined.

Attention should further be directed to the results obtained with the hydrochloride of the methylurethane of *m*-dimethylaminophenol. Reference to Table VI will show that 0·0001 mg. of this substance produced, at p_H 7·9, an inhibition of the activity of the liver esterase of approximately 50 %. Since, in the final reaction mixture, the inhibitor was contained in a volume of about 50 cc., the final concentration of this substance required to diminish the activity of the esterase by this amount was about 1:500,000,000. This is the greatest activity which has been observed with any of the urethanes so far examined. It agrees with other results obtained with the same urethane, which has, in fact, proved to be the most active inhibitor amongst the three series of compounds investigated.

Comparison of the inhibitory activities of isomeric urethanes. In order to obtain a direct comparison of the activities of the members of a given series of urethanes, it was necessary, for reasons stated in connection with the similar experiments carried out with methyl butyrate as substrate, to measure the inhibitory activities on the same enzyme preparation. The results are recorded in Table VII. Different esterase solutions were employed for the three series, so that the figures recorded do not necessarily give a comparison of the activities of members of different series. In the benzyl and phenyl series, an interval of some days elapsed between the experiments with the hydrochlorides and methiodides. The enzyme had meanwhile undergone slight deterioration, as shown by the figures for the second control.

The order of inhibitory activity of the various isomerides on liver esterase, using tributyrin as substrate, is, according to the results of Table VII as follows.

Miotine series. The hydrochlorides do not show large differences in activity. Nevertheless they appear to be definitely in the order $m > o > p$. With the methiodides, which are considerably less active, the *o*-compound exhibits the greatest activity; the *m*- and *p*-isomerides are, within the limits of experimental error, equally active.

Benzyl series. The hydrochlorides of this series show greater differences in activity, the order being quite definitely $o > m > p$. The methiodides again show little difference in activity; the order $p > o > m$ is indicated by the results.

Table VII. *Comparison of the inhibitory activities of isomeric urethanes.*

Substrate: tributyrin. T=20°. p_H=7·9.

Inhibitor	Final conc. of inhibitor (molar × 10^{-7})	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Miotine series:	Control	11	22	—
m-HCl	40	0	2	91
,,	4	5	10	56
p-HCl	40	3·5	7·5	68
,,	4	6	12	47
o-HCl	40	1	3·5	86
,,	4	5	10·5	52
m-MeI	400	2	3	87
p-MeI	400	2	4	83
o-MeI	400	0	1·5	91
Benzyl series:	Control	12	23	—
m-HCl	400	1	2·5	87
,,	40	8	16	30
p-HCl	400	4	8·5	61
,,	40	10	20	13
o-HCl	400	0	1	96
,,	40	3	6	84
	Control	11·5	21	—
m-MeI	400	7	15	29
p-MeI	400	6	12	43
o-MeI	400	6·5	13·5	38
Phenyl series:	Control	13	27	—
m-HCl	4	1	2	90
p-HCl	4	5	10	63
o-HCl	4	5	10·5	59
	Control	11·5	21	—
m-MeI	40	8	16	24
p-MeI	40	8	16	24
o-MeI	40	3	7	67

Phenyl series. In this series the *m*-hydrochloride is considerably more active than the *o*- and *p*-compounds, which can scarcely be distinguished from one another in activity. Of the methiodides, the *o*-compound is the most active, the *m*- and *p*-isomerides possessing the same activity. It should be noted that the order of inhibitory activity of both the hydrochlorides and methiodides of this series is identical with that of the miotine series.

Discussion.

The foregoing results have established definitely that urethanes of the type which have been shown in earlier papers to possess very specific physiological properties also possess the common property of inhibiting in low concentration the activity of liver esterase. Of the nine urethanes examined, all exhibit this property to a marked degree, and not only do they do so in the form of their hydrochlorides but their methiodides are similarly, although usually somewhat less, active. It appears legitimate to conclude that a relationship exists between chemical constitution and inhibitory activity towards liver esterase analogous to that between constitution and physiological action in the same group. In conformity with this conclusion, the methylurethane of choline

iodide, a substance containing many structural features in common with the active urethanes but lacking the essential phenyl group, is inactive. Similarly, the hydrochloride of *α-m*-hydroxyphenylethyldimethylamine, the phenol from which miotine is prepared and which contains the phenyl and basic but not the urethane group, produces no inhibition in relatively high concentration.

Whether these results hold for the enzyme, present in blood-sera from certain species and in tissue extracts, which is normally responsible for the destruction of acetylcholine, has not yet been determined. Nevertheless, the fact that urethanes of the type which possess physiological properties similar to those of physostigmine also inhibit, in minute concentration, the hydrolysis by liver esterase of a simple ester such as methyl butyrate, affords substantial support to the view that the above-mentioned serum-enzyme, the activity of which towards acetylcholine has been shown by Loewi and his co-workers and by Matthes to be inhibited by physostigmine, is a true esterase such as is present in the liver and is not one which acts specifically towards acetylcholine.

It is not proposed to discuss in detail the question, which now arises, as to whether the physiological activity of urethanes of the miotine type is an indirect result of the inhibitory action which they exert upon esterases, since this problem has already been considered from many aspects by White and Stedman [1931], who have pointed out that while much of the activity of miotine, in particular its toxicity, may probably be attributed to its inhibition of the destruction of acetylcholine, other mechanisms are possibly also involved. One fact, which supports the suggestion, originally made by Loewi and Navratil [1926], that the action of physostigmine on the heart is due to its inhibitory action on the acetylcholine-destroying enzyme, may, however, be mentioned. It has been shown above that arecoline, in relatively high concentration, does not inhibit the activity of liver esterase. Now this alkaloid belongs, pharmacologically, to the physostigmine group of drugs and might therefore be expected to resemble physostigmine in its behaviour towards esterases. That it does not do so is in complete accord with some unpublished pharmacological experiments by Dr A. C. White, to whom we are indebted for information regarding his results, according to which arecoline, unlike physostigmine, does not potentiate the action of acetylcholine on the vagus.

It is not to be expected, even on the assumption that the whole of the physiological activity of urethanes of the miotine type is attributable to the resulting accumulation of acetylcholine in the organism, that an exact parallelism will exist between the magnitude of their physiological activities and that of their inhibitory activities towards esterases, for the drugs will be subject to many influences in the organism which are eliminated in *in vitro* experiments with enzymes. Thus, a property of a drug, such as adsorbability, which may render it particularly efficient in inhibiting the activity of an esterase, may actually prevent it from reaching the enzyme in the organism. Similarly, stability will be of greater influence in *in vivo* than in *in vitro* experiments, since in the former case the drug is subjected to the action of

more destructive agents. That no such parallelism does, in fact, exist follows from our experiments; the order of the inhibitory activity of various urethanes towards liver esterase in no way corresponds with the previously published provisional order for their miotic activities.

Perhaps the most interesting questions which call for consideration in connection with our experiments are those concerning the nature of the inhibitory action and its bearing on the mechanism of the normal action of esterases. Of a group of compounds possessing a common structural feature, every member which has been examined has been found to inhibit, in minute concentration, the activity of liver esterase. These inhibitory substances, like the normal substrate of the enzyme, are esters. Nevertheless they are either not attacked, or are only hydrolysed extremely slowly, by the esterase. The suggestion thus arises that the urethanes combine with the esterase in the same way and by means of the same mechanism as do simple esters, but that the affinity between urethane and enzyme is enormously greater than that between simple ester and enzyme. Hence the esterase-urethane compound is formed preferentially and, owing to the inability of the enzyme to decompose the urethane, the hydrolysis of the normal substrate is prevented. The latent period which occurs in the hydrolysis of ethyl mandelate by liver esterase when esters of certain keto-acids are present has been similarly explained by Willstätter, Kuhn, Lind and Memmen [1927], and further experiments in support of this explanation have been published by Bamann and Schmeller [1930]. On the above basis the relative inhibitory activities of the various urethanes will be a measure of their affinities for the esterase, and any regularity which is observed between inhibitory and other properties should give a clue as to the nature of the affinity between the urethanes and esterase, and hence of the nature of the forces normally responsible for the formation of enzyme-substrate compound. Now, the following regularities have, in fact, been noted in our experiments.

(1) In a given series of urethanes the isomerides show inhibitory activities which, although usually different, are of the same order of magnitude.

(2) Of the three series examined, the hydrochlorides of the phenyl series are outstanding with respect to their high inhibitory activities.

(3) The methiodides are, in general, considerably less active than the hydrochlorides of the corresponding tertiary bases.

(4) The inhibitory activity of a given urethane is considerably smaller at p_H 6·8 than in slightly alkaline medium.

We believe that these results can be best interpreted by assuming that the affinities in question are, in the first instance, of the nature of adsorptive forces. If we postulate (*a*) that the urethane group confers high adsorbabilities on the inhibitory substances, (*b*) that the adsorbability and therefore inhibitory activity is increased by any factor which tends to lower the solubility, (*c*) that the free bases and not the salts are the active inhibitors, our reasons for this belief will be at once clear. In accordance with postulates (*b*) and (*c*),

any factor which tends to convert the readily soluble salts into the sparingly soluble bases, thus diminishing the solubility and increasing the concentration of the active constituent, would be expected to assist adsorption and hence increase inhibitory activity. Increase in alkalinity is such a factor, and as shown above this increases the inhibitory activity. Similarly diminishing the basic strength of the compounds would constitute another such factor; corresponding with this the compounds which are the weakest bases, namely, the methylurethanes of the phenyl series, show the greatest inhibitory activity. Further, solubility is increased by converting the tertiary bases into their quaternary ammonium salts, the free bases of which are readily soluble in water. This should diminish adsorbability and hence inhibitory activity, as is actually the case.

Nevertheless, adsorption is clearly not the only process involved. The possession of surface activity of a high order will not necessarily confer upon a compound the marked inhibitory activity shown by the above urethanes. Moreover, it has been shown by Willstätter, Kuhn and Bamann [1928] that esters of *d*- and *l*-mandelic acids exhibit different affinities towards esterases, while Murray and King [1930] have demonstrated that certain enantiomorphic alcohols inhibit the activity of liver esterase to different degrees. Optical isomerides would be expected to possess identical adsorbabilities; it is therefore almost certain that some factor other than adsorption is also involved. We are of opinion that, following adsorption, the inhibitor enters into especial relation with the enzyme, the exact nature of which is not at present clear, and it is probably this second stage of the combination process which is the cause of the different inhibitory activities of isomeric urethanes. One fact which has been elicited in our experiments tends to support this view. In examining the inhibitory activities of the various urethanes towards liver esterase, it has been found that the general effect of those factors which we have interpreted above as exerting their influence by producing changes in adsorbability is the same whether the substrate is methyl butyrate or tributyrin. The relative activities of isomeric urethanes vary, however, with the nature of the substrate, a result which cannot be explained on the basis of adsorption.

Summary.

1. Urethanes of the type which have previously been shown to possess physiological properties of the same kind as physostigmine have been found to possess the common property of inhibiting the activity of liver esterase. A relationship thus exists between chemical constitution and inhibitory action towards liver esterase analogous to that between constitution and physiological activity in the same group.

2. This inhibitory action is exerted in high dilution. Thus, the hydrochloride of the *m*-dimethylaminophenyl ester of methylcarbamic acid, which

is the most active urethane examined, produced an inhibition of 50 % in the hydrolysis of tributyrin by liver esterase when in a final concentration of about 1:500,000,000. The same urethane inhibited the hydrolysis of methyl butyrate to about the same extent when in a final concentration of $\frac{1}{3} \times 10^{-6} M$, which corresponds with one of about 1:13,000,000.

3. Three series of isomeric urethanes, which have been termed the methylurethanes of the phenyl, benzyl and miotine series respectively, have been examined for inhibitory activity in the forms both of their hydrochlorides and methiodides. The relative activities of the isomerides, when methyl butyrate is employed as substrate, are as follows.

Hydrochlorides. Phenyl series: $m > p > o$; benzyl series: $m > p > o$; miotine series: $m = p > o$.

Methiodides. Phenyl series: $m \gtrless p > o$; benzyl series: $p > o > m$; miotine series: $o > p \gtrless m$.

Using tributyrin as substrate the following orders, which differ from those above, are obtained:

Hydrochlorides. Phenyl series: $m > p \gtrless o$; benzyl series: $o > m > p$; miotine series: $m > o > p$.

Methiodides. Phenyl series: $o > p = m$; benzyl series: $p > o > m$; miotine series: $o > p = m$.

4. The inhibitory activity is, in every case, greater in slightly alkaline medium than at p_H 6·8.

5. In general, the methiodides exert a considerably smaller inhibitory effect than do the hydrochlorides.

6. The relative inhibitory activities of the various urethanes do not correspond with their relative miotic activities. It is pointed out that such correspondence is not to be expected even on the assumption that the whole of the physiological activity of the urethanes is due to their inhibitory action on esterases.

7. The mechanism of the inhibitory action is discussed.

Part of the expense of this investigation has been met by grants from the Earl of Moray Research Fund of this University.

REFERENCES.

Bamann (1929). *Ber. deutsch. chem. Ges.* **62**, 1538.
—— and Schmeller (1930). *Z. physiol. Chem.* **188**, 251.
Engelhart and Loewi (1930). *Arch. exp. Path. Pharm.* **150**, 1.
Galehr and Plattner (1927). *Pflüger's Arch.* **218**, 488, 507.
Loewi and Navratil (1926). *Pflüger's Arch.* **214**, 689.
Matthes (1930). *J. Physiol.* **70**, 338.
Murray and King (1930). *Biochem. J.* **24**, 190.
Plattner and Galehr (1928). *Pflüger's Arch.* **220**, 606.
Rona and Bach (1920). *Biochem. Z.* **111**, 166.
—— and Michaelis (1911). *Biochem. Z.* **31**, 345.

Stedman (1926). *Biochem. J.* **20**, 719.
—— (1929, 1). *Biochem. J.* **23**, 17.
—— (1929, 2). *Amer. J. Physiol.* **90**, 528.
—— and Stedman (1929). *J. Chem. Soc.* 609.
—— —— (1931). *J. Chem. Soc.* 1126.
White and Stedman (1931). *J. Pharm. Exp. Ther.* **91**, 259.
Willstätter, Bamann and Waldschmidt-Graser (1928). *Z. physiol. Chem.* **173**, 155.
—— Kuhn and Bamann (1928). *Ber. deutsch. chem. Ges.* **61**, 886.
—— —— Lind and Memmen (1927). *Z. physiol. Chem.* **167**, 303.
—— and Memmen (1923). *Z. physiol. Chem.* **129**, 1.
—— —— (1924, 1). *Z. physiol. Chem.* **133**, 229.
—— —— (1924, 2). *Z. physiol. Chem.* **138**, 216.

CXXX. THE GLYCERIDE STRUCTURE OF BEEF TALLOWS.

By ADAM BANKS and THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received June 30th, 1931.*)

In continuation of the studies of the constitution of different classes of natural fats which have occupied our attention for some time, an examination has been made of four samples of beef tallow which varied in composition within fairly wide limits. With the exception of a specimen of mutton tallow [Collin, Hilditch and Lea, 1929], body fats from the ox or sheep have not hitherto been submitted to complete investigation by the present methods.

Bömer and his co-workers [1907, 1909], by repeated fractional crystallisation of the solid glycerides present in tallow, established the presence of a number of simple and mixed glycerides. Thus, these workers state that beef tallow contains 0·5–1·5 % of tristearin and varying proportions of α-palmitodistearin, stearodipalmitin, oleodipalmitin and oleopalmitostearin, and that about 3 % of tristearin, 4–5 % of α-palmitodistearin, 4–5 % of stearodipalmitin, were accompanied in mutton tallow by larger quantities of oleodipalmitin and oleopalmitostearin. In the specimen of mutton tallow investigated by the present methods, Collin, Hilditch and Lea [1929] found 26 % of fully-saturated compounds which consisted for the most part of mixed glycerides of palmitic and stearic acid; fractional crystallisation of the fully-saturated components indicated the probable presence of about 3 % of tristearin, 9 % of palmitodistearin and 6–7 % of dipalmitostearin, leaving about 7–8 % of the original fat to be accounted for as tripalmitin and mixed glycerides of myristic, palmitic and stearic acids.

In the present work we have not carried out any fractional crystallisation of the solid components of the tallows, since we desire rather to discuss the general composition of the fats from a quantitative standpoint, in a similar manner to the recent treatment of the component glycerides of butter fat by Hilditch and Sleightholme [1931].

The beef tallows employed in the present work were from American consignments kindly selected for us by Messrs Lever Bros., Ltd., and consisted of a North American tallow (N) and three samples of South American premier jus which will be referred to respectively as SA, SB and SC. Each material thus represents bulk samples typical of different centres of production rather than fat from a single animal or even from separate herds. This was desirable from the original motive of this particular investigation, which was to establish

the main characteristics of market supplies of beef tallows. As the work progressed certain general features became apparent, *viz.* that, as in the butter fats [Hilditch and Sleightholme, 1931], (i) the composition of the fully-saturated glycerides tends to be constant irrespective of their proportion in the whole fat, and (ii) the combined amounts of stearic, oleic and linoleic acids in the whole fats also tend towards a constant value, irrespective of wide variations in the respective amounts of stearic and of unsaturated (oleic and linoleic) acids.

The saponification equivalents and iodine values of the specimens examined were as follows:

Table I.

Beef tallow	M.P. (open tube)	Saponification equivalent	Iodine value	Setting-point of mixed fatty acids
N	42°	284·2	49·5	42·3°
SA	46°	280·5	42·1	45·9°
SB	47·5°	284·0	37·1	47·2°
SC	47·5°	285·7	39·3	47·0°

Component fatty acids of the four beef tallows.

The composition of the mixed fatty acids from the tallows was determined according to the methods which we have described on several occasions in some detail [*cf.* Hilditch and Jones, 1929, 1931]. The mixture of fatty acids (palmitic, stearic, oleic with minor amounts of myristic and linoleic) is comparatively simple in this case, and, after preliminary separation into the usual groups of "solid" and "liquid" acids by the lead salt and alcohol separation, the fractionation of the corresponding group of methyl esters presents no great difficulty. The final results of the analyses only are tabulated below but, in order to illustrate the method of calculation employed, the fractionation data for one of the tallows (N) are reproduced in Table II.

In calculating the results, it is assumed (i) that the only unsaturated acid present in the esters of the "solid" acids is oleic, and (ii) that the mixture of unsaturated esters distilling over throughout from the esters of the "liquid" acids has the same composition as that of the pure unsaturated ester fraction (L 8 in Table II) finally obtained. The mean saponification equivalents of the saturated esters in each fraction are determined after making allowance for those of the unsaturated esters present.

Some trouble was experienced with the onset of rancidity in the original fats and, by the time specimens SB and SC were analysed, this had developed

Table II. *Fatty acids of beef tallow N.*

	Lead salt separation.		Corresponding esters	
	g.	%	Sap. eq.	I.V.
"Solid" acids S	113	46·5	278·2	7·0
"Liquid" acids L	130	53·5	287·5	80·71
	243			

Table II (*contd.*).

Fractionation and estimated composition of methyl esters.

(i) *Esters of "solid" acids S.*

No.	g.	B.P./1 mm.	Sap. eq.	I.V.	C_{14}	C_{16}	C_{18}	Ol.
S 1	4·68	114 –129°	264·3	2·7	1·00	3·53	—	0·15
S 2	6·24	129 –128	265·9	3·0	1·00	5·02	—	0·22
S 3	5·47	128 –130	268·1	3·2	0·47	4·80	—	0·20
S 4	9·53	130 –132	270·5	3·8	0·14	8·97	—	0·42
S 5	9·53	132 –132·5	271·5	4·1	—	8·79	0·29	0·45
S 6	10·24	132·5–133	275·5	4·6	—	8·00	1·69	0·55
S 7	10·10	133 –136	278·1	5·8	—	6·89	2·53	0·68
S 8	10·07	136	280·8	7·3	—	5·91	3·30	0·86
S 9	9·76	136 –138	284·4	9·6	—	4·43	4·24	1·09
S 10	8·57	138 –141	289·6	11·7	—	2·32	5·08	1·17
S 11	11·19	141 –146	295·8	13·0	—	0·65	8·85	1·69
S 12	5·37	Residue	297·5	14·8	—	0·06	4·38	0·93
				Weights	2·61	59·37	30·36	8·41
				% esters	2·6	59·0	30·1	8·3
				% acids	2·6	58·8	30·2	8·4

(ii) *Esters of "liquid" acids L.*

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	36·94	71–137·5°	273·8	69·4
L 2	11·63	137·5	285·5	82·5
L 3	14·11	137·5–144	291·7	85·0
L 4	9·11	144	292·9	86·3
L 5	9·55	144–145	292·6	86·3
L 6	10·46	145–144	293·8	88·2
L 7	8·88	144	294·0	89·0
L 8	8·58	144–147	296·3	90·2
L 9	8·65	Residue	320·6	88·6

Refractionations

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 11	3·34	73–105°	244·5	40·7
L 12	5·68	105–110	256·1	53·0
L 13	3·53	110–118	270·1	64·8
L 14	3·45	118–128	279·4	72·8
L 15	3·97	128–130	284·0	76·3
L 16	3·64	130–135	288·2	79·9
L 17	3·27	135	290·2	83·4
L 18	4·52	Residue	291·6	86·6

No.	g.	as C_{14}	C_{16}	Oleic	Linoleic	Unsap.
L 1	36·94	8·58	—	28·36		—
L 2	11·63	0·99	—	10·64		—
L 3	14·11	0·82	—	13·29		—
L 4	9·11	0·25	0·14	8·72		—
L 5	9·55	0·42	—	9·13		—
L 6	10·46	0·23	—	10·23		—
L 7	8·88	0·12	—	8·76		—
L 8	8·58	—	—	8·58		—
L 9	8·65	—	—	7·99		0·66
Weights		11·41	0·14	100·33	5·37	0·66
% esters		9·7	0·1	85·1	4·6	0·5
% acids		9·6	0·1	85·2	4·6	0·5

No.	g.	as C_{14}	Oleic	Linoleic
L 11	3·34	1·83	1·51	
L 12	5·68	2·34	3·34	
L 13	3·53	0·99	2·54	
L 14	3·45	0·67	2·78	
L 15	3·97	0·61	3·36	
L 16	3·64	0·42	3·22	
L 17	3·27	0·25	3·02	
L 18	4·52	0·18	4·34	
Weights		7·29	24·11	
% esters		23·2	76·8	

to an appreciable extent. In consequence the values obtained for the linoleic acid contents of these fats are almost certainly lower, and those for myristic acid higher, than the truth owing to the presence of traces of low-boiling products of decomposition which it is not yet possible accurately to correct for in the course of the analysis. A summary of the analytical data obtained for the mixed fatty acids from each tallow is given in Table III.

Table III. *Summarised experimental data for fatty acids of beef tallows.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	Fatty acids (excluding unsaponifiable matter) (% mols.)
		Beef tallow N.			
	(46·5 %)	(53·5 %)			
Myristic	1·20	5·12	6·32	6·3	7·5
Palmitic	27·35	0·06	27·41	27·4	29·1
Stearic	14·06	—	14·06	14·1	13·4
Oleic	3·89	45·57	49·46	49·6	47·6
Linoleic	—	2·44	2·44	2·5	2·4
Unsaponifiable	—	0·31	0·31	—	—
		Beef tallow SA.			
	(54·0 %)	(46·0 %)			
Myristic	0·43	4·03	4·46	4·5	5·3
Palmitic	30·56	—	30·56	30·6	32·9
Stearic	19·04	—	19·04	19·1	18·1
Arachidic	0·12	—	0·12	0·1	0·1
Oleic	3·85	38·78	42·63	42·7	40·7
Linoleic	—	2·96	2·96	3·0	2·9
Unsaponifiable	—	0·23	0·23	—	—
		Beef tallow SB.			
	(59·0 %)	(41·0 %)			
Myristic	1·83	6·00	7·83	7·8	9·5
Palmitic	27·70	—	27·70	27·8	29·2
Stearic	24·37	—	24·37	24·4	23·2
Oleic	5·10	33·67	38·77	38·9	37·1
Linoleic	—	1·08	1·08	1·1	1·0
Unsaponifiable	—	0·25	0·25	—	—
		Beef tallow SC.			
	(57·7 %)	(42·3 %)			
Myristic	1·26	4·40	5·66	5·8	6·9
Palmitic	23·68	—	23·68	24·0	25·5
Stearic	28·22	—	28·22	28·6	27·4
Oleic	4·54	36·48	41·02	41·6	40·2
Unsaponifiable	—	1·42	1·42	—	—

Fully-saturated glyceride contents of the four beef tallows.

Each fat was submitted, in quantities of 100 g. at a time, to oxidation in ten times its weight of acetone by means of four times its weight of powdered potassium permanganate. The procedure has been recently described in detail by Hilditch and Sleightholme [1931]. The results obtained are given in detail in Table IV.

Table IV. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) *Analytical data.*

			Purification of crude fully-saturated glycerides							
				"A"		"B"		Acidic products		
No.	Weight taken g.	Crude F.S.G. g.	Weight taken g.	g.	Acid No.	g.	Acid No.	g.	Acid No.	F.S.G. % (wt.)
SB	399·8	117·5	115·6	85·6	0·5	15·7	28·5	14·3	270·0	25·3
SC	204·4	56·0	54·7	42·9	0·4	9·2	25·1	2·6	232·7	25·6
SA	316·0	86·4	84·8	39·4	0·7	32·9	22·3	12·2	278·9	22·5
N	604·2	92·0	81·0	39·5	0·8	35·2	10·4	6·3	167·7	13·6

(ii) *Summary of observed values.*

		Acids in whole fat		Fully-saturated glycerides		
No.	Iodine value	Saturated % (mol.)	Unsaturated % (mol.)	Weight %	Mols. %	"Association ratio" in non-fully-saturated part. Mols. saturated acid per mol. unsaturated acid
SB	37·1	61·9	38·1	25·3	25·8	0·94
SC	39·3	59·8	40·2	25·6	26·0	0·84
SA	42·1	56·4	43·6	22·5	22·8	0·75
N	46·6	50·0	50·0	13·6	13·9	0·72

The purified fully-saturated glycerides "A" (*cf.* Table IV) were converted into their mixed fatty acids, and thence into the corresponding mixed methyl esters, which were fractionally distilled in the usual manner. Since these analytical results have led us to conclude that there is a considerable approach to constancy in the composition of the fatty acids of the fully-saturated components of the tallows, it may be well to record the fractionation data in full (Table V).

Table VI illustrates the general composition of 100 parts by weight of the four tallows for the whole fats, for the fully-saturated glyceride portions, and (by difference) for the portions of the fat consisting of mixed saturated-unsaturated and (possibly) fully-unsaturated glycerides.

Discussion.

Before proceeding to consider the fatty acids present in the whole tallows and in their component subdivisions of fully-saturated and non-fully-saturated glycerides, attention may be directed to the general relationships subsisting between the fully-saturated glyceride content and the total content of saturated acids in each tallow. The range of unsaturation encountered in these fats is not relatively so great as that which was available in the series of butter fats [1931], but nevertheless it suffices, we believe, to show that the same kind of relationship subsists in the beef tallows as in cow milk fats. Moreover, the data formerly obtained [Collin, Hilditch and Lea, 1929] for a mutton tallow also display the same general features and, although we do not wish to minimise the differences which in many cases characterise fats from different species or genera of animals or plants, the body fats of the sheep and ox

Table V. *Fractionation of methyl esters from fully-saturated glycerides.*

Fraction	g.	B.P./1 mm.	Sap. eq.	Myristate g.	Palmitate g.	Stearate g.	Unsap. g.
			Fully-saturated glycerides of beef tallow SB.				
1	3·68	121–131°	262·4	0·92	2·76	—	—
2	4·32	131–132	266·8	0·45	3·87	—	—
3	4·28	132–134	272·4	—	3·87	0·41	—
4	3·23	134–140	277·3	—	2·33	0·90	—
5	6·20	140–141	279·6	—	3·93	2·27	—
6	8·26	141–145	286·1	—	3·31	4·95	—
7	5·25	145	289·0	—	1·58	3·67	—
8	5·75	Residue	304·3	—	0·77	4·78	0·20
	40·97		Weights	1·37	22·42	16·98	0·20
			% esters	3·3	54·7	41·5	0·5
			% acids	3·3	54·6	41·6	0·5
			Fully-saturated glycerides of beef tallow SC.				
1	3·76	104–128°	263·0	0·86	2·90	—	—
2	4·44	128–130	268·1	0·27	4·17	—	—
3	4·88	130–135	274·1	—	4·10	0·78	—
4	3·79	135–137	280·7	—	2·25	1·54	—
5	3·34	137–140	286·3	—	1·32	2·02	—
6	3·27	140	289·8	—	0·89	2·38	—
7	5·32	Residue	305·7	—	0·47	4·67	0·18
	28·80		Weights	1·13	16·10	11·39	0·18
			% esters	3·9	55·9	39·6	0·6
			% acids	3·9	55·8	39·7	0·6
			Fully-saturated glycerides of beef tallow SA.				
1	1·43	104–120°	247·8	1·11	0·32	—	—
2	1·55	120–129	259·5	0·54	1·01	—	—
3	3·31	129–125	266·6	0·36	2·95	—	—
4	2·85	125–123	271·6	—	2·67	0·18	—
5	2·52	123–119	275·8	—	1·96	0·56	—
6	2·94	119–116	282·8	—	1·52	1·42	—
7	10·21	Residue	294·6	—	3·41	6·57	0·23
	24·81		Weights	2·01	13·84	8·73	0·23
			% esters	8·1	55·8	35·2	0·9
			% acids	8·0	55·7	35·3	1·0
			Fully-saturated glycerides of beef tallow N.				
1	2·39	104–117°	258·3	0·94	1·45	—	—
2	3·65	117–124	265·0	0·60	3·05	—	—
3	3·06	124–127	272·6	—	2·75	0·31	—
4	2·94	127–132	277·0	—	2·15	0·79	—
5	3·67	132–136	281·7	—	2·05	1·62	—
6	4·07	136–140	287·2	—	1·48	2·59	—
7	5·05	Residue	309·1	—	0·38	4·45	0·22
	24·83		Weights	1·54	13·31	9·76	0·22
			% esters	6·2	53·6	39·3	0·9
			% acids	6·2	53·5	39·4	0·9

happen not to be very dissimilar in composition and it may be useful to include the data for the specimen of sheep tallow formerly examined[1].

In the case of the butter fats we showed that there was a parallelism between the molar proportion of fully-saturated glycerides and (i) the proportion of saturated and unsaturated acids in the whole fat, and (ii) the corresponding molar proportion of fully-saturated glycerides in synthesised

[1] The mutton tallow is designated in the tables by (M).

Table VI. *General composition (weights) of beef tallows.*

	Whole fat	Fully-saturated	Non-fully-saturated
	Beef tallow SB.		
	100 g.	25·3 g.	74·7 g.
Glyceryl residue $C_3H_2\equiv$	4·45	1·15	3·3
Myristic acid	7·45	0·8	6·65
Palmitic ,,	26·55	13·25	13·3
Stearic ,,	23·3	10·1	13·2
Oleic ,,	37·2	—	37·2
Linoleic ,,	1·05	—	1·05
	Beef tallow SC.		
	100 g.	25·6 g.	74·4 g.
Glyceryl residue $C_3H_2\equiv$	4·4	1·2	3·2
Myristic acid	5·5	1·0	4·5
Palmitic ,,	23·0	13·7	9·3
Stearic ,,	27·3	9·7	17·6
Oleic ,,	39·8	—	39·8
	Beef tallow SA.		
	100 g.	22·5 g.	77·5 g.
Glyceryl residue $C_3H_2\equiv$	4·5	1·0	3·5
Myristic acid	4·3	2·0	2·3
Palmitic ,,	29·2	11·7	17·5
Stearic ,,	18·3	7·8	10·5
Oleic ,,	40·8	—	40·8
Linoleic ,,	2·9	—	2·9
	Beef tallow N.		
	100 g.	13·6 g.	86·4 g.
Glyceryl residue $C_3H_2\equiv$	4·4	0·6	3·8
Myristic acid	6·0	0·8	5·2
Palmitic ,,	26·3	7·0	19·3
Stearic ,,	13·5	5·2	8·3
Oleic ,,	47·4	—	47·4
Linoleic ,,	2·4	—	2·4

mixed glycerides of corresponding total unsaturation, or (iii) of the molar proportion of fully-saturated glycerides calculated on the basis of proportionality to the cube of the total saturated acids present. At the same time the "association ratio" of saturated and unsaturated acids in the non-fully-saturated glycerides varied correspondingly with the total unsaturation. Similar relationships for the four beef tallows and the mutton tallow (M) are shown graphically in Fig. 1, in which the appropriate proportions of the synthetic glyceride curve (unbroken line) of Bhattacharya and Hilditch [1930] and of the "calculated" curve (dotted) are plotted together with the observed values for the tallows.

This group of tallows therefore affords further confirmation of the generalisation that in animal fats the nature of the mixed glycerides is mainly determined by the relative proportions of saturated and unsaturated acids present. In the body fats and milk fats of cattle, for any given ratio of saturated to unsaturated acids, the mode of assemblage in the triglyceride molecules appears to follow a more or less fixed course with the general result that the

amount of fully-saturated glycerides is somewhat greater than that proportionate to the cube of the total saturated acid content.

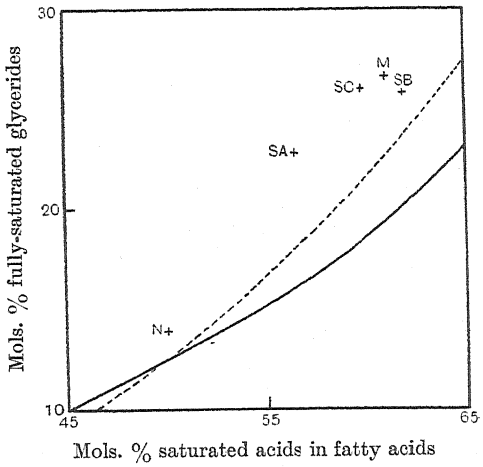


Fig. 1.

The molar association ratios lead to limiting values for the possible proportions of either mono-unsaturated-disaturated and diunsaturated-monosaturated glycerides, or mono-unsaturated-disaturated and tri-unsaturated glycerides which may be present, and the approximate data in question are collected in Table VII. The actual proportion of tri-unsaturated glycerides present is uncertain; the presence of tristearin and (probably) of tripalmitin to a total extent of perhaps 5–8 % of the whole fat has been indicated by fractional crystallisation of the mixed glycerides (*cf.* p. 1168). This indicates a somewhat greater tendency to the production of simple triglycerides than in the butter fats, and, by analogy, there may be some likelihood of the presence of triolein (in amount not exceeding, perhaps, some 10 % of the whole fat). No quantitative means for the determination of triolein being yet available, it is not possible to do more than assign the extreme possible limits of the three classes of unsaturated glycerides quoted in the Table.

Table VII. *Limiting values for the molar percentages of the four types of mixed glycerides in the fat.*

			Glycerides (mols. %)			
Tallow	Iodine value	"Association ratio"	Fully-saturated	Mono-unsaturated-disaturated	Diunsaturated-mono-saturated	Tri-unsaturated
SB	37·1	0·94	25·8	33·7–53·9	40·5–0	0–20·3
(M)	41·2	0·90	26·6	30·9–52·1	42·5–0	0–21·3
SC	39·3	0·84	26·0	27·3–50·7	46·7–0	0–23·3
SA	42·1	0·75	22·8	22·1–49·6	55·1–0	0–27·6
N	49·5	0·72	13·9	22·0–54·1	64·1–0	0–32·0

Distribution of individual fatty acids in the glycerides of tallows.

The discussion of the component fatty acids of the tallows will be based throughout on the proportionate number of molecules of the different fatty acids present, that is, on the molar percentages (and not the weight percentages) of the fatty acids. In Table VIII will be found respectively the molar distribution of fatty acids (1) in the whole tallows, (2) in their fully-saturated glycerides, and (3) in their mixed saturated-unsaturated glycerides; the fats are conveniently dealt with in decreasing order of fully-saturated glyceride content.

Table VIII.

	(M)	SC	SB	SA	N
Molar distribution of fatty acids.					
(1) Whole fat:					
Myristic acid	5·5	6·9	9·5	5·3	7·5
Palmitic ,,	26·2	25·5	29·2	32·9	29·1
Stearic ,,	29·3	27·4	23·2	18·2	13·4
Oleic ,,	34·8	40·2	37·1	40·7	47·6
Linoleic ,,	4·2	—	1·0	2·9	2·4
(2) Fully-saturated glycerides:					
Molar % F.S.G.	26·6	26·0	25·8	22·8	13·9
Myristic acid	7·1	4·5	3·9	9·3	7·2
Palmitic ,,	52·1	58·2	57·0	57·7	55·8
Stearic ,,	40·8	37·3	39·1	33·0	37·0
(3) Mixed saturated-unsaturated glycerides:					
Molar % M.S.-U.	73·3	74·0	74·2	77·2	86·1
Myristic acid	4·9	7·8	11·1	3·7	7·5
Palmitic ,,	16·9	13·9	19·8	25·4	24·8
Stearic ,,	25·0	23·9	17·7	13·7	9·6
Oleic ,,	47·5	54·4	50·0	53·4	55·3
Linoleic ,,	5·7	—	1·4	3·8	2·8

As in the case of the butter fats, considerable interest attaches to comparison of the proportion of C_{18} (stearic, oleic and linoleic) acids present with the total proportion of the remaining acids (palmitic with small amounts of myristic). The molar percentages of these groups present in each fat and in its two subdivisions are shown in Table IX.

Table IX. *Molar distribution of myristic-palmitic and C_{18} acids in the tallows.*

Section	Fat ... / Acids	(M)	SC	SB	SA	N
Whole fat	Myristic-palmitic	31·7	32·4	38·7	38·2	36·6
	C_{18} acids	68·3	67·6	61·3	61·8	63·4
	(Stearic	29·3	27·4	23·2	18·2	13·4)
Fully-saturated	Myristic-palmitic	59·2	62·7	60·9	67·0	63·0
	Stearic	40·8	37·3	39·1	33·0	37·0
Saturated-unsaturated	Myristic-palmitic	21·8	21·7	30·9	29·1	32·3
	C_{18} acids	78·2	78·3	69·1	70·9	67·7
	(Stearic	25·0	23·9	17·7	13·7	9·6)
	(Oleic-linoleic	53·2	54·4	51·4	57·2	58·1)

Component fatty acids of the whole fats.

Table VIII (1) illustrates the general composition of the fatty acids of the tallows which we have studied, and shows that oleic, palmitic and stearic acids are the predominating components, accompanied by relatively small amounts of linoleic acid and of myristic acid[1]. The values for palmitic acid are the most constant in the group, and in this respect resemble the corresponding data for palmitic acid in butter fats; indeed, they suggest, like those of the butter fats, that palmitic acid on the one hand and the C_{18} acids on the other probably have little in common so far as their mode of synthesis in the plant or animal is concerned.

Our meaning will perhaps be clearer if Table IX is consulted, and the molar percentages of the C_{18} acids in the whole tallows are considered. The only remaining acids are palmitic and myristic, which are given together (palmitic acid accounting for 70 %, or usually more, of this group). The five specimens now under consideration (including the mutton tallow M) fall into two clearly defined groups when classified in this way: specimens M and SC contain about 68 mols. % of C_{18} acids, and the remaining three about 61–63 mols. %, although throughout the five fats the molar proportion of saturated C_{18} (stearic) acid varies from 13 to 29 mols. %. Taking the group as a whole, the total content of C_{18} acids thus tends much more towards a more or less steady value than does the content of the stearic acid included therein.

It is interesting to add to the foregoing data the following earlier analyses of tallow mixed fatty acids given by Armstrong and Allan [1924]. Their figures, reduced to molar percentages as in Tables VIII and IX, are quoted in Table X.

Table X.

[From Armstrong and Allan, 1924.]

	Beef			Mutton	
	North American	South American	Australian	South American	Australian
Myristic acid	2·4	3·0	2·4	1·8	2·4
Palmitic „	34·5	26·7	28·3	22·5	26·8
Stearic „	13·9	19·3	21·7	29·0	22·2
Oleic „ / Linoleic „	49·2	51·0	47·6	46·7	48·6
Myristic-palmitic	36·9	29·7	30·7	24·3	29·2
C_{18} acids	63·1	70·3	69·3	75·7	70·8
(Stearic	13·9	19·3	21·7	29·0	22·2)

It will be seen that, of these five specimens, three contain 69–71 % of C_{18} acids and one 63 %, whilst the fifth sample is definitely out of line with either group.

Thus out of nine samples of beef and mutton tallows which have been submitted to detailed analysis five contain about 70 mols. % of C_{18} acids with

[1] It should be recalled that in specimens SB and SC the myristic acid figures given are probably slightly high, and those for linoleic acid somewhat low, owing to the onset of rancidity as mentioned on p. 1171.

about 30 mols. % of palmitic (with myristic) acid, and four contain about 62 % of C_{18} acids with about 38 % of palmitic (with myristic) acid—the stearic acid molar content meantime varying from 13 to 29 %. Whilst much further study of tallows from specific herds or animals would probably be necessary to settle the point, there is some indication here that two classes of mixed fatty acids may occur, in both of which the relative amounts of C_{18} and the remaining acids are almost constant; although relative proportions vary somewhat between one group and the other, both are marked by a characteristic proportion of total C_{18} acids irrespective of wide variations in the subordinate proportions of saturated (stearic) and unsaturated (oleic with linoleic) acids.

The constitution of the tallow mixed glycerides.

If the component fatty acids of the fully-saturated parts of the fats are considered under the headings of stearic and palmitic (with myristic), as in Table IX, it will be observed that the molar percentage of stearic acid lies between 37 and 41 in the case of four out of the five tallows, the remaining one (SA) being slightly lower (33). On comparing these values for stearic acid with those in the whole fats, it is evident that the composition of the fully-saturated glycerides tends to be much the same however greatly the proportion of stearic acid present in the whole of the fat varies (13–29 mols. %), and whatever the fully-saturated glyceride content (14–26 %) of the fat[1].

This tendency towards constant composition of the fully-saturated portions of the fat also appeared very clearly in the parallel investigation of butter fats [Hilditch and Sleightholme, 1931], and we conclude that the component fatty acids of the fully-saturated part of an animal fat are to a large extent characteristic and definite for a given species.

It is also of some interest to note that the molar proportion of palmitic (with myristic) acid in one group of the whole tallows (37–38 %) is much the same as that recently observed in a number of butter fats (34–37 %), whilst in the corresponding fully-saturated glycerides the usual figure in the tallows appears to be about 60 % as compared with 55–58 % for the milk fats. It may further be observed that, whereas, as already mentioned, the C_{18} acid molar content of the whole tallows seems to lie in one of two groups (*ca.* 62 % and *ca.* 68 %), this distinction disappears in their fully-saturated glycerides, where the usual range is 37–41 %.

So far as the non-fully-saturated glycerides are concerned, it follows arithmetically that the molar contents of the two groups of acids, palmitic (with myristic) and total C_{18}, will show the same resemblance as those in the whole fats: namely, two groups in which, within narrow limits, approximate constancy is again observed (palmitic with myristic acid respectively *ca.* 22 and *ca.* 30—total C_{18} acids respectively *ca.* 78 and *ca.* 70 %). The variation

[1] The fully-saturated glycerides isolated from the four beef tallows melted respectively at 54° (SA), 54·5° (SB), 54·5° (SC) and 54° (N).

in the stearic acid molar content (9·6–25·0 %) is, of course, more pronounced than in the fats as a whole since differences in the amount of stearic acid in the whole tallows are concentrated entirely in the non-fully-saturated portion of the fats.

This feature is perhaps rendered more clear by tabulating the relative proportions of stearic and palmitic (with myristic) acids in the whole fats and in the non-fully-saturated portions, and comparing the percentage values obtained (Table XI) with those for the fully-saturated glycerides in Table IX:

Table XI.

Relative proportions of myristic-palmitic and stearic acids in whole fats.

	(M)	SC	SB	SA	N
Myristic-palmitic	52·0	54·2	62·5	67·7	73·2
Stearic	48·0	45·8	37·5	32·3	26·8

Relative proportions of myristic-palmitic and stearic acids in the non-fully-saturated glycerides.

	(M)	SC	SB	SA	N
Myristic-palmitic	46·6	47·6	63·6	68·0	77·1
Stearic	53·4	52·4	36·4	32·0	22·9

The widely varying proportions in the whole fat are reduced to something approaching constancy in the fully-saturated glycerides and correspondingly accentuated in the non-fully-saturated glycerides.

It may also be remarked that the "association ratio" with respect to stearic acid in the fully-saturated glycerides is usually approximately 1·5 mols. of palmitic (with myristic) acid per mol. of stearic acid, a value which, curiously, is the same as that in which acids present in major proportions are usually found linked with acids present in minor proportions in vegetable seed fats; it is not likely, however, that this connotes any great similarity in mode of assemblage, since the presence of definite though small amounts of tristearin and tripalmitin indicates that the distribution of the fatty acids is less even in the fully-saturated parts of animal fats than in seed fats.

The foregoing results lead to the conclusion that the body fats of the ox (and probably also the sheep) are made up of a mixture of fully-saturated components, tending towards constancy in composition, with non-fully-saturated glycerides in which the amounts of stearic and oleic acids may vary considerably, but that the sum of these two acid components is nevertheless more or less constant and typical. These general tendencies must be intimately connected with the metabolic processes which are involved in the deposition of storage fat. It is difficult to draw conclusions as to the nature of such processes from an examination of their end-products, but the following points arising out of the above data may be noticed.

1. The consistency or melting-point of a body fat is generally agreed to be more or less adapted to the environment of the animal, and it has usually

been defined hitherto as being determined by the proportion of liquid or unsaturated fatty acids which are present. The present data suggest further that variation in consistency is obtained by fluctuation in the relative amounts of stearic and oleic acids, subject to the condition that the united proportion of these acids remains approximately constant. Also, the melting-point of the fat is determined by the presence of varying amounts of relatively high-melting fully-saturated glycerides of approximately constant composition accompanied by inversely varying proportions of glycerides containing oleic acid, which will tend to be lower or higher in melting-point according to the presence of less or more stearic acid in the approximately constant amount of total C_{18} acids.

2. The manner in which the fatty acids are assembled into body fats constituted in conformity with these generalisations forms an interesting subject for discussion. The well-established fact that animal fats are assembled in an essentially different manner from vegetable seed fats (although not very differently from fruit-flesh fats and, therefore, possibly, from leaf fats) reinforces the accepted view that ingested fats are hydrolysed and resynthesised into glycerides in the animal. Thus, assuming that a given mixture of palmitic, stearic, oleic and linoleic acids is available for laying down as depot fat, it appears that the glyceride synthesis operates in such a way that there is an approximately constant molar proportion of stearic and oleic (total C_{18}) acids in the product; at the same time, a certain amount of glycerides containing only stearic and palmitic (with myristic) acids is laid down and this portion always contains about 37–40 % of stearic residues. The approximately constant balance between the C_{18} and the C_{16} (*plus* C_{14}) acids in the whole fat, despite varying stearic acid content in the latter, is then maintained by variation in the amount of unsaturated acid in the non-fully-saturated portions, and also in the relative amounts of fully-saturated and non-fully-saturated glycerides.

So far as is known at present, the components of vegetable fats normally ingested by cattle are relatively small amounts of palmitic acid with smaller but variable amounts of stearic acid accompanied by considerable proportions of oleic acid and, usually, still more linoleic acid[1]. It is conceivable that from such a mixture of fatty acids glycerides may be laid down in conformity with the generalisations concerning their structure at which we have arrived above, the available palmitic and stearic acids being combined so as to give the more or less constant proportions of palmitic acid in the whole fats by inclusion of the requisite amount of oleic and linoleic acids to balance the stearic acid which is also utilised. In the tallows the amount of oleic and linoleic acids is definitely lower than in the plant oils which we visualise as being the main source of the fatty acids assimilated by the animal, and there must remain an excess of these after the body fat has been synthesised, and this excess

[1] The recent work of Barber [1929] has shown that mixtures of these four acids are also produced from carbohydrate material by ferment action.

may well be disposed of by oxidation; some such method of elimination of the excess of linoleic acid appears to be necessary, moreover, to account for the characteristic and relatively small ratio of linoleic to oleic acid in the tallows, and also for the quantitatively similar ratio in the milk fats.

It should be emphasised, also, that the relative proportion of stearic to palmitic acid in the whole tallows is as a rule much higher than that in any fatty component likely to be present in the diet of cattle, and that therefore we are faced either with the possibility that palmitic acid is destroyed, or with the more likely alternative that stearic acid is synthesised, either from corresponding unsaturated acids or from non-fatty material, in the animal organism. In either of these latter alternatives, it appears that a process of "saturation" rather than "desaturation" predominates on the whole during the elaboration of tallows, and we consider, with Leathes and Raper [1925], that "desaturation" appears unlikely as a process which operates in the synthesis of unsaturated acids previous to their storage as neutral fat.

We hope to obtain further insight into the problems discussed above from a parallel study, at present in progress, of the body-fats of the pig, in which we are investigating the fats from a single animal fed on a controlled diet.

Summary.

Four beef tallows from various North and South American sources have been investigated with reference to their content of fully-saturated glycerides and the component fatty acids of the latter and of the whole fats.

In the whole fats the united molar percentage of stearic, oleic and linoleic acids tends towards one of two definite figures (*ca.* 62 or *ca.* 69 mols. %) in spite of wide variations in the amount of stearic, and correspondingly of oleic (with linoleic), acid. This relation is also shown to subsist in a number of other tallows for which previous analyses are available.

The proportion of fully-saturated glycerides present varies according to the relative total amounts of saturated and unsaturated acid in the whole fats, but the composition of the acids present therein tends to much the same figure in all cases, namely, about 60 % of palmitic (with myristic) acid and about 40 % of stearic acid.

These regularities in structure, which are strikingly similar to those recently observed in a series of butter fats, are discussed in regard to their possible implications concerning the processes involved in the elaboration of storage fat in the animal.

We desire to thank Messrs Lever Bros., Ltd., for selection and provision of the materials employed in this study, and the Department of Scientific and Industrial Research for a grant to one of us (A. B.) during its progress.

REFERENCES.

Armstrong and Allan (1924). *J. Soc. Chem. Ind.* **43**, 216 T.
Barber (1929). *Biochem. J.* **23**, 1158.
Bhattacharya and Hilditch (1930). *Proc. Roy. Soc. Lond.* A **129**, 468.
Bömer, Schemm and Heimsoth (1907). *Z. Unters. Nahr. Genussm.* **14**, 90.
—— and Heimsoth (1909). *Z. Unters. Nahr. Genussm.* **17**, 353.
Collin, Hilditch and Lea (1929). *J. Soc. Chem. Ind.* **48**, 46 T.
Hilditch and Jones (1929). *Analyst*, **54**, 75.
—— —— (1931). *J. Soc. Chem. Ind.* **50**, 171 T.
—— and Sleightholme (1931). *Biochem. J.* **25**, 507.
Leathes and Raper (1925). The fats, 2nd edition, p. 120. (Longmans.)

CXXXI. DETERMINATION OF INORGANIC SULPHATE IN HUMAN BLOOD-PLASMA BY MICRO-TITRATION.

By CUTHBERT LESLIE COPE[1].

From the Department of Biochemistry, Oxford.

(*Received June 30th, 1931.*)

Observations on the inorganic sulphate content of human blood and other body fluids are relatively infrequent, partly owing to the great difficulties encountered in estimating the small concentrations of inorganic sulphate present therein, but also because the sulphate ion has been regarded as pharmacologically inert and as therefore of little importance in the elucidation of physiological and pathological problems. That it is pharmacologically inert is probably true. The intravenous injection of sodium sulphate into human beings in amounts up to 3 or 4 g. certainly has no detectable effects save an increase in the amount of sulphate excreted in the urine[2], and Sollman [1926] states that much larger amounts may be given without demonstrable effects.

There are however certain problems in the solution of which a knowledge of the blood sulphate would be of considerable value. Peters and his associates [1929] have called attention to the probable rôle of sulphate in accounting in part for the "undetermined acid" combining with base in cases of nephritis with nitrogen retention and have made a plea for further investigation of its behaviour in the organism.

The suggestion that sulphate, being apparently merely filtered off by the renal glomeruli and not again re-absorbed in the tubules, could be used as a measure of the volume of glomerular filtrate [White, 1923; Rehberg, 1926; Sager, 1930; Poulsson, 1930], has greatly increased the need for a satisfactory method of estimation of inorganic sulphate in small quantities of blood-plasma or serum. Rehberg considered the estimation too difficult in plasma. White and also Sager, simplified the problem by artificially increasing the blood sulphate concentration to more easily estimated levels, whilst Poulsson contented himself with a rough estimate of the probable concentration in the blood during his experiments.

Numerous methods for plasma inorganic sulphate estimation have from time to time been described. The more recent of these may be classified as follows.

(*a*) *Gravimetric*, Meyer-Bisch [1924], Pohorecka-Lelesz [1927], Loeb and Benedict [1927].

[1] Beit Memorial Research Fellow.

[2] Personal observation, unpublished.

(*b*) *Nephelometric*, Denis [1921], Denis and Reed [1926], Chatron [1931].

(*c*) *Benzidine, by titration*, White [1923], Sager [1930], Pohorecka-Lelesz [1927], Chatron [1931], Lesure and Dunez [1929].

(*d*) *Benzidine, colorimetric*, Yoshimatsu [1926], Wakefield [1929], Hubbard [1930].

(*e*) *Sulphide formation*, Lorant [1929].

(*f*) *Miscellaneous*, Lang [1929].

Of these different methods, the gravimetric requires either a large volume of blood or very accurate weighing on a micro-balance. The nephelometric method suffers from the fact that the turbidity of the suspension of barium sulphate is very dependent on changes in the p_H and composition of the blood-filtrate. The titration of benzidine sulphate has, so far as the writer is aware, only been applied to blood in which sulphate concentration has been artificially raised, or in which it is already higher than in human blood [Pohorecka-Lelesz, 1927].

The colorimetric benzidine processes require great care in their manipulation if colour is to remain proportional to sulphate content. The colours obtained, moreover, being weak yellows and browns, are difficult to compare accurately, at least for some workers.

Reduction to a sulphide is a somewhat laborious procedure and appears to include ethereal and other sulphates in the analysis. Finally, the method of Lang, whilst giving excellent pink colours, has not, in the hands of the writer at least, given constant results with such low concentrations of sulphate as are encountered in normal human plasma.

Numerous attempts have been made by the writer to improve on one or other of these methods. A modification of the Denis and Reed nephelometric procedure and a micro-titration method based on Hubbard's technique [1930] will be described.

Nephelometric method.

Denis's claim for an accuracy of 5 % for her modified method [1926] has been fully confirmed for calf's and sheep's serum in both of which the actual inorganic sulphate concentration is five or six times as high as in human serum. Table I shows the results obtained.

Table I. *Comparison of gravimetric and Denis nephelometric methods.*

Serum used	Gravimetric S mg./100 cc.	Nephelometric S mg./100 cc.	Error (%)
Calf's serum	5·29	5·36	1
,, ,,	7·16	6·94	3
Sheep's serum	3·59	3·73	4
,, ,,	3·51	3·73	6
Calf's serum	4·77	4·58	4
,, ,, (with added S)	5·25	5·45	4
,, ,, (diluted 1 : 1)	2·38	2·46	1
Sheep's serum	3·12	3·06	2
,, ,, (with added S)	3·74	3·71	1

When however the same method was applied to human plasma or serum, the results were far less satisfactory. Denis herself stated that, if from a blood-filtrate no precipitate is obtained with barium chloride, it is necessary before concluding that no sulphate is present to determine the p_H of the solution, the optimum value for which she gives as 3·0 to 3·8. The method as described by Denis, however, takes no account of the p_H of the final filtrate, but assumes that in all ordinary cases this will be such as to give a reliable nephelometric reading, if the reagent quantities given are adhered to.

It was felt that herein lay a possible source of the discrepant results, since, if an unfavourable p_H may completely prevent the precipitation of barium sulphate, then one somewhat less unfavourable may evidently seriously affect the resultant turbidity without necessarily arousing suspicion. This was found actually to be the case, and a p_H range of from 1·8 to 2·4 was found to yield maximum and fairly constant turbidities. This is rather more acid than the p_H recommended by Denis and slightly more so than that suggested by Chatron, but the small differences may be due to variations in the gelatin stabilisers employed.

It was, however, found that even with a constant p_H, the total concentration of trichloroacetic acid present had a serious effect on the turbidity produced. This is well shown in the following example in which a solution with a sulphate content corresponding to the filtrate from a plasma with 0·65 mg. S per 100 cc. was employed.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·1	0·25	0·5	0·75	1·0	1·5
Final p_H to thymol blue	2·5	2·4	2·4	2·3	2·4	2·2
Percentage error in estimation	6	3	6	20	28	43

The presence of small quantities of trichloroacetic acid in the solution did not materially influence the result. The excess can be conveniently removed by shaking the protein-free blood-filtrate with two volumes of ether, if allowance be made for the resultant change in volume of the water phase (about 7 %). Much more consistent results are then obtained, as is shown in the following example, in which the same concentration of sulphate as above was used.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·25	1·5	1·5	1·5
Final p_H to thymol blue	2·0	2·0	2·0	2·0
			Extracted with ether	
Percentage error in estimation	1	32	1	3·5

The same effect was observed with other low concentrations of sulphate.

It is evident therefore that in order that the nephelometric method should yield even approximately constant results with human plasma, (*a*) the p_H of the solution must be controlled in every case and brought to the optimum value; (*b*) the quantity of trichloroacetic acid present in the filtrate must be small.

The presence of ether in the extracted solution appeared to have little effect on the nephelometric readings, but it is desirable to treat the standard sulphate solutions in a similar manner.

Denis's method modified in this manner was employed for some time by the writer with fairly satisfactory results but was finally abandoned in favour of the micro-titration benzidine technique. As this has proved itself to be in every way preferable to the modified nephelometric method, details of technique of the latter will not be given.

Both procedures give figures varying from 0·5 to 1·5 mg./100 cc. of S as inorganic sulphate for normal human plasma and thus agree with the now generally accepted values.

Micro-titration benzidine method.

This is a modification of the method advocated by Hubbard [1930] and indeed follows his procedure in detail as far as the production of the washed benzidine sulphate precipitate. This Hubbard estimated colorimetrically, but estimation by direct titration has been found more reliable for low sulphate concentrations. For convenience, Hubbard's procedure is described here. For further details the original paper should be consulted.

Reagents.

(*a*) 1 % benzidine in good acetone.
(*b*) 20 % sulphate-free trichloroacetic acid in water.
(*c*) Good grade acetone.
(*d*) *N*/50 CO_2-free sodium hydroxide.

Method.

To 2 to 5 cc. of serum or citrated plasma in a centrifuge tube is added an exactly equal volume of 20 % trichloroacetic acid, and the whole is thoroughly mixed with a thin glass rod. The precipitated proteins are centrifuged, leaving a clear supernatant fluid which usually requires no further filtration.

In a 10 cc. Pyrex glass centrifuge tube with sharply pointed tip are placed 5 cc. of the 1 % benzidine in acetone solution, and to this are added exactly 2 cc. of the protein-free blood-centrifugate. The tube is stoppered and kept in ice-water for an hour, or until flocculation of the precipitate begins to occur. It is centrifuged and drained for 5 minutes, the tube being inverted in a small beaker in the bottom of which is a pad of filter-paper moistened with acetone. This prevents evaporation of the acetone before drainage is complete. 10 cc. of fresh acetone are added, the precipitate is broken up with a fine glass rod and centrifuged; the tube is again drained on the pad for 5 minutes.

Titration. For this purpose the micro-burette described by Rehberg [1925] has been found eminently suitable. It should be filled with *N*/50 CO_2-free sodium hydroxide. The titration is carried out in the Pyrex centrifuge tube itself, this being surrounded by a steam-jacket. The apparatus recommended is shown in Fig. 1.

The jacket is readily made from ordinary glass. Steam enters by the side-tube from a suitable flask, and escapes with the condensed water into a

receiver placed below the bottom tube. The upper opening carries a rubber stopper bored with a hole into which the centrifuge tube fits.

Mixing of the solution during titration is effected by bubbling through it a slow stream of CO_2-free air, most conveniently obtained from two large bottles connected by a syphon and containing weak sodium hydroxide solution. The rate of bubbling is controlled by a screw-clip.

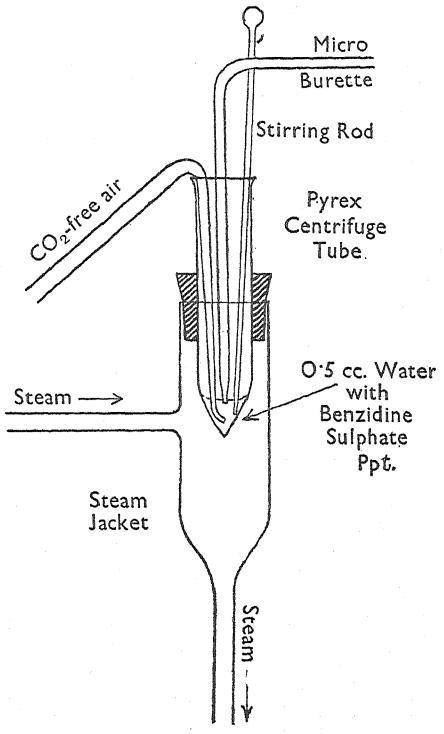


Fig. 1.

To the tube containing the washed and drained benzidine sulphate precipitate is added 0·5 cc. of distilled water. Phenolphthalein is added by dipping the tip of a fine glass rod into the indicator solution and inserting it into the centrifuge tube, where it may be allowed to remain, as it is useful for breaking up the precipitate during the titration. The centrifuge tube with its fine glass stirring rod is inserted into the steam-jacket, and the whole arranged so that the tip of the microburette just dips under the surface of the liquid in the tube. The tube for bubbling CO_2-free air is next placed in position and a gentle stream commenced.

It is advisable to carry the titration as far as possible before the steam is turned on. When the pink colour due to the added alkali tends to become persistent, the steam is turned on and the titration completed as rapidly as possible at 100°. No difficulty is encountered in detecting the first appearance of a permanent pink if good lighting be arranged. Before removing the tube its sides are well washed down by turning on the air supply fully for a moment, and further alkali is added if necessary. The centrifuge tube and jacket are then immediately removed from the tip of the burette. Some haste is desirable during these later stages in order to minimise the heating of the fluid in the burette during the titration. After the tube is withdrawn cooling leads to a slight retraction of fluid from the tip of the burette. The meniscus must be brought once again to the tip by turning the control screw, before the final reading of the burette is made.

A blank titration is necessary in order to determine the amount of alkali required to produce a definite pink colour in 0·5 cc. of distilled water at 100°. This usually lies between 3·0 and 4·5 mm.^{3} of $N/50$ sodium hydroxide, and the blank must be subtracted from the titration figure.

This procedure has given more reliable results than any other tried by the writer. The figures in Table II indicate the degree of accuracy obtained.

All the determinations were performed on samples of sheep's serum which had been dialysed for 14 days against pure 0·9 % sodium chloride. It is interesting that, in the second specimen of serum, sulphate in a concentration of 0·06 mg. S per 100 cc. could be definitely detected, and produced a distinctly visible deposit of benzidine sulphate .

Table II.

Number	Added S mg./100 cc.	Theoretical S mg./100 cc.	Added citrate mg./100 cc.	Added P mg./100 cc.	mm³ $N/50$ sodium hydroxide used (corrected)	Estimated S mg./100 cc.	(%) recovery
1	0·83	0·83	—	—	26·1	0·836	100
2	1·15	1·15	—	—	38·2	1·22	108
3	1·15	1·15	—	—	35·1	1·12	98
4	1·43	1·43	—	—	43·3	1·38	96
5	1·43	1·43	—	—	43·2	1·38	96
6	1·37	1·37	200	—	41·4	1·32	96
7	1·37	1·37	200	—	41·3	1·32	96
8	1·67	1·67	—	—	52·5	1·68	100·6
9	1·67	1·67	—	—	52·0	1·665	100
10	1·62	1·62	200	—	51·6	1·65	102
11	1·62	1·62	200	—	49·0	1·565	96·6
12	2·14	2·14	—	—	69·0	2·20	103
13	2·14	2·14	—	—	67·3	2·15	100·5
14	1·67	1·67	—	3·3	53·4	1·71	102·4
15	1·67	1·67	—	3·3	52·9	1·69	101·2
16	1·36	1·36	—	6·5	41·6	1·33	97·8
17	1·36	1·36	—	6·5	43·0	1·375	100·7
Fresh dialysed serum estimated to contain 0·06 mg. S per 100 cc.							
18	2·31	2·37	—	—	76·0	2·44	103
19	2·31	2·37	—	—	77·0	2·46	104
20	0·91	0·97	—	10·0	30·4	0·97	100
21	0·91	0·97	—	10·0	32·1	1·02	105
22	1·67	1·73	—	10·0	54·5	1·74	100·6
23	1·67	1·73	—	10·0	55·2	1·76	102
24	0·55	0·615	—	12·3	20·1	0·64	104
25	0·55	0·615	—	12·3	18·6	0·595	96·7
26	2·00	2·06	—	22·0	74·0	2·37	115
27	2·00	2·06	—	22·0	74·0	2·37	115
28	0·45	0·515	—	10·0	16·4	0·525	102
29	0·45	0·515	—	10·0	17·2	0·55	107
Third sample of dialysed serum free of sulphate.							
30	0·91	0·91	900	5·0	28·2	0·905	99·5
31	0·91	0·91	900	5·0	27·9	0·896	98·6
32	2·86	2·86	900	5·0	101·0	3·23	112
33	2·86	2·86	900	5·0	97·0	3·11	108
34	3·33	3·33	900	5·0	111·0	3·56	107
35	3·33	3·33	900	5·0	114·0	3·64	109
36	3·85	3·85	900	5·0	129·0	4·14	107·5
37	3·85	3·85	900	5·0	130·0	4·16	108

The figures show that even a large excess of citrate has no appreciable effect on the estimation.

Concentrations of phosphate up to 12 mg. P per 100 cc. of original plasma have no effect on the results, and the figures show a satisfactory agreement with the theoretical up to about 2·5 mg. S per 100 cc. but tend to be a little too

high when the sulphate concentration exceeds that value, and much phosphate is present.

SUMMARY.

Some errors in the nephelometric method of estimating inorganic sulphate in small amounts of human plasma or serum are considered, and means of overcoming them are suggested.

Details of a modified benzidine technique are given in which the more usual colorimetric estimation is replaced by micro-titration with sodium hydroxide. With the filtrate from 1 cc. of human serum or plasma this method gives results within 5 % of the theoretical.

The author wishes to express his thanks to Prof. R. A. Peters for many helpful suggestions, and to Dr J. G. Priestley for the loan of Rehberg microburettes.

REFERENCES.

Chatron (1931). *Bull. Soc. Chim. Biol.* **13**, 300.
Denis (1921). *J. Biol. Chem.* **49**, 311.
—— and Reed (1926). *J. Biol. Chem.* **71**, 191.
Hubbard (1930). *J. Biol. Chem.* **88**, 663.
Lang (1929). *Biochem. Z.* **213**, 469.
Lesure and Dunez (1929). *Bull. Soc. Chim. Biol.* **10**, 879.
Loeb and Benedict (1927). *J. Clin. Invest.* **4**, 33.
Lorant (1929). *Z. physiol. Chem.* **185**, 245.
Meyer-Bisch (1924). *Biochem. Z.* **150**, 23.
Peters, Wakeman, Eisenman and Lee (1929). *J. Clin. Invest.* **6**, 517.
Pohorecka-Lelesz (1927). *Bull. Soc. Chim. Biol.* **9**, 263.
Poulsson (1930). *Z. ges. exp. Med.* **71**, 577.
Rehberg (1925). *Biochem. J.* **19**, 270.
—— (1926). *Biochem. J.* **20**, 447.
Sager (1930). *Arch. exp. Path. Pharm.* **153**, 331.
Sollmann (1926). Manual of pharmacology, p. 824. (Philadelphia.)
Wakefield (1929). *J. Biol. Chem.* **81**, 713.
White (1923). *Amer. J. Physiol.* **65**, 537.
Yoshimatsu (1926). *Tohoku J. Exp. Med.* **7**, 119.

CXXXII. THE IODINE METHOD FOR THE DETERMINATION OF GLUTATHIONE.

By NICOLAE GAVRILESCU.

From the Department of Biochemistry, Oxford.

(*Received June 30th, 1931.*)

The usual and more practical method for the estimation of glutathione in the tissue extracts is considered to be Tunnicliffe's iodine method [1925]. The end-point of the reaction is determined by the use of either starch or sodium nitroprusside as indicator. In both cases there are some difficulties in determining the true end-point. With the starch as internal indicator the end-point is very uncertain; the initial blue colour, once attained, persists for an interval of time varying from 5 to 30 seconds with the different tissue extracts, and this time of persistence increases with the amount of iodine added after reaching the initial blue colour. With very pure samples of glutathione the end-point is much more easy to determine with precision. It is probable that this trouble is introduced by the presence in the extracts of foreign substances which react with the iodine with different speeds so that the final figures will always be larger and will not represent the true amount of the glutathione present in the tissue extracts.

The sodium nitroprusside end-point is more accurate and the difficulty in using it consists only in appreciating the point of disappearance of the red colour.

All authors have remarked on the discrepancy between the figures obtained, using starch or nitroprusside as indicator.

Voegtlin and Thompson [1926] believe that the starch is not specific enough as indicator. Kennaway and Hieger [1927], in their experiments, found large differences (from 40 to 50 %) between starch and nitroprusside end-points. Bierich and Kalle [1928] came to the same conclusions. Furthermore, Mason [1930] assumes that the method is not only subject to the error introduced by these modes of determining the end-point, but even the iodine estimation itself is not trustworthy for some tissue extracts, for example: liver, kidney and yeast. He compared the iodine method with a colorimetric one, based on the oxidation of the .SH group in glutathione with potassium ferricyanide. Mason gives a table (IV) showing results which are very suggestive, but it is difficult to understand the wide variation in the individual figures in his estimation of glutathione from yeast, using nitroprusside as external indicator. In the present experiments, using the same sample of yeast extract, the numerous estimations performed with nitroprusside as indicator, have always

given very consistent results, provided that the nitroprusside test is performed under very accurate and standardised conditions, as suggested for instance by Kennaway and Hieger [1927]

Considering the difficulties in obtaining concordance in the results of glutathione estimation from tissue extracts with the starch-iodine method, Perlzweig and Delrue [1927] proposed a modification of Tunnicliffe's method consisting in addition of an excess of 25 % potassium iodide (2 cc. for each sample ready for estimation). According to their view the potassium iodide stabilises the end-point; this is also the experience of the present author. However, the potassium iodide introduces another error if we do not take into account the acidity of the sample.

The present experiments are therefore a comparative study of the estimation of glutathione in pure solutions of this substance and in tissue extracts using one of the following indicators.

(1) Starch in ordinary acid extracts.
(2) Starch, acid solution and 2 cc. of 25 % potassium iodide.
(3) Starch *plus* potassium iodide in sample neutral to litmus.
(4) Nitroprusside.

The results are shown in the Tables.

Table I. *Glutathione estimations in various organs.*

For each estimation 10 cc. of extract.

	cc. $N/100$ iodine			
		2 cc. of 25 % KI		
	No KI; acid	Acid	Neutral to litmus*	Nitro-prusside end-point
Sheep brain†; approx. 50 g. tissue for 150 cc. extract	1·50	0·56	0·86	0·80
	1·56	0·54	0·84	0·80
	1·54	0·54	0·84	0·80
	1·54	0·54	—	0·80
Beef; approx. 50 g. tissue for 150 cc. extract	0·7	0·36	0·54	0·50
	0·7	0·32	0·56	0·50
	0·7	0·36	0·50	0·50
	0·7	0·36	0·54	—
Yeast; approx. 50 g. for 250 cc. extract	1·44	1·16	1·40	1·44
	1·44	1·18	1·42	1·46
	1·44	1·14	1·44	1·44
	1·44	1·14	1·42	1·46
	1·44	1·16	—	—
Liver; approx. 50 g. tissue for 250 cc. extract	2·46	0·86	1·1	1·2
	2·42	0·82	1·1	1·2
	2·46	0·82	1·2	1·2
	2·40	0·82	1·1	1·2
Glutathione 0·225 %. Very pure sample. For each estimation 1 cc. in 10 cc. of 10 % trichloroacetic acid	0·92	0·76	0·88	0·9
	0·92	0·76	0·9	0·9
	0·90	0·78	0·86	0·9
	0·90	0·78	0·88	—

* The neutralisation was effected by addition of $2N$ NaOH.

† These figures do not represent the real amount of the glutathione present in tissues because the latter have been washed only two or three times.

To avoid any misunderstanding it is necessary to consider some of the major points of the method.

The tissue extracts have been made with pure (Merck) trichloroacetic acid (10 %). The filtrate was clear. The potassium iodide solution was prepared by dissolving a pure sample in freshly boiled and cooled water. Fresh solutions have always been used and were placed in the dark immediately after preparation. The nitroprusside test was performed according to the suggestions given by Kennaway and Hieger [1927].

Table II. *The effect of varying concentrations of KI in acid and neutral solutions.*

	cc. of 25 % KI	cc. $N/100$ iodine: Acid	cc. $N/100$ iodine: Neutral
Liver	0·0	1·84 and 1·84	1·1 and 1·1
	0·1	1·3 „ 1·34	1·06 „ 1·1
	0·2	1·08 „ 1·08	—
	0·5	0·92 „ 0·94	—
	1·0	0·82 „ 0·82	1·1 „ 1·08
	2·0	0·68 „ 0·68	—
	3·0	—	1·06 „ 1·08
Glutathione 0·225 %	0·0	0·90	0·88
	0·1	0·82	0·86
	0·2	0·82	0·88
	0·5	0·82	0·88
	1·0	0·80	0·86
	2·0	0·76	0·88
	3·0	0·70	0·84 and 0·86

Table III. *The effect of* p_H *on the glutathione estimation in the presence of excess of potassium iodide.*

Glutathione 0·25 % (for each estimation 1 cc. glutathione solution *plus* 10 cc. phosphate mixture *plus* 2 cc. 25 % KI).

						Strongly acid (control)	
p_H	5·9	6·46	6·8	6·9	7·16	KI	No KI
cc. iodine $N/100$	0·80	0·84	0·88	0·94	1·00	0·8	1·0
	0·82	0·84	0·88	0·94	1·00	0·8	1·0
	0·80	0·84	—	0·92	—	0·8	—

We notice that the depressant action of the potassium iodide is present even with samples of very pure glutathione (Table I).

Table IV. *Sheep brain.*

70 g. for 250 cc. extract; comparative estimation of the glutathione in acid or neutral solution, with or without KI.

Acid			Neutral	
No KI	2 cc. of 25 % KI	Nitroprusside end-point	No KI	2 cc. of 25 % KI
1·34	0·50	0·74	1·22	0·76
1·38	0·52	0·78	1·16	0·8
1·42	0·50	0·76	1·2	0·78
1·38	—	0·74	1·2	—
—	—	—	1·2	—

Discussion.

This error introduced by the presence of potassium iodide was noticed by Okuda [1925] in his experiments on the estimation of cysteine. He believed that the discrepancies might be explained by assuming different chemical reactions between cysteine and iodide under the different conditions of estimation, but he did not prove this point experimentally. However, he correlated the difference in the reaction with the temperature, acidity and presence of potassium iodide. To avoid the effect of these different factors which interfere with the results, he proposed for the cysteine estimation specially standardised conditions.

If we consider now the figures from the present paper, we notice the following very suggestive facts.

In the estimation of pure samples of glutathione there is no difference between starch-iodide (no KI) and nitroprusside end-points.

The same statement holds for the yeast samples. The same comparison for beef, brain and liver extracts gives respectively the average differences 29 %, 47 %, and 50 %. Do these differences between starch and nitroprusside end-points represent some foreign substances interfering with the estimation of the glutathione? Probably, but if so, why are there no differences between these figures when the estimation with starch as indicator is made in neutral solution?

Indeed we notice from our Tables that, if the nitroprusside end-point in the estimation of .SH groups is the most specific one, the iodine-starch-KI method may be very accurate, provided we take into account some special condition with regard to the acidity of the sample.

In acid solution the use of excess KI produces lower figures than the nitroprusside. Near, or just at the neutral point of the solution, the figures obtained with excess potassium iodide are concordant with the nitroprusside figures (Tables I and II).

If the estimation is made at the neutral point but without adding potassium iodide in excess, the figures obtained are higher than those indicated by nitroprusside. Table IV shows this comparative experiment. Therefore the KI seems to be necessary for a good estimation.

Table III is a good demonstration of the relations between the p_H and the estimation of pure glutathione when the KI is present in excess.

The depressant action of KI in acid solution depends also upon its concentration (see Table II), but by neutralising the sample this effect of concentration is eliminated. What is the explanation of this fact?

First we must remember that in the ordinary estimation of sodium thiosulphate with iodine, the addition of KI in acid solution in no way affects the end-point, using starch as indicator. A fundamental difference between the thiosulphate and the glutathione estimations seems to be that in the former there is no liberation of HI, whereas HI is liberated in the latter. The trouble may thus be introduced by the formation of the HI which in the presence of

KI reaches some equilibrium with the liberation of free iodine. That the amount of free iodine is correlated with the p_H of the sample is suggested by our experimental results (see Table II).

We can consider a second explanation. In acid solution, in the presence of certain compounds contained in the tissue extracts, the KI could liberate iodine, and the free iodine would then react with the .SH groups to some extent before the titration was commenced. This view is not supported by the following experimental fact. If we leave some glutathione or extract in the presence of 2 cc. of 25 % KI, estimating then the glutathione at different time intervals, we find no difference between the figures.

The most probable explanation is the physico-chemical explanation of equilibrium between KI-HI-I, this equilibrium being correlated with the p_H of the sample. Further investigation will perhaps indicate how the equilibrium is to be formulated.

Conclusions.

A comparative study has been made of glutathione estimation in tissue extracts, either with starch or nitroprusside as indicators.

In comparison with the nitroprusside end-point the starch in acid samples and without KI gives very high figures. In the presence of excess of KI on the contrary, lower results are obtained.

The figures obtained in the estimation of glutathione in pure solution and in tissue extracts, using excess of potassium iodide and making the sample neutral to litmus, are concordant with the figures obtained, using the nitroprusside end-point.

The author proposes the above modification of Tunnicliffe's method which is very accurate when compared with the methods employing nitroprusside as an indicator.

I wish to express my sincere gratitude to Prof. R. A. Peters for his criticism, and to thank Dr Dixon for some samples of pure glutathione.

REFERENCES.

Bierich and Kalle (1928). *Z. physiol. Chem.* **175**, 115.
Kennaway and Hieger (1927). *Biochem. J.* **21**, 751.
Mason (1930). *J. Biol. Chem.* **86**, 623.
Okuda (1925). *J. Biochem.* **5**, 201.
Perlzweig and Delrue (1927). *Biochem. J.* **21**, 1416.
Tunnicliffe (1925). *Biochem. J.* **19**, 194.
Voegtlin and Thompson (1926). *J. Biol. Chem.* **70**, 793.

CXXXIII. THE FATE OF CAROTENE AFTER ABSORPTION IN THE ANIMAL ORGANISM.

By BASHIR AHMAD.

From the Department of Physiology and Biochemistry, University College, London.

(*Received July 1st, 1931.*)

THERE is now ample evidence that administration of carotene to animals fed on a vitamin A-deficient diet is followed by a return to normal health and the appearance in the liver of a substance showing the properties of vitamin A [Moore, 1929, 1930, 1931; Drummond, Ahmad and Morton, 1930; Capper, 1930; Capper, McKibbin and Prentice, 1931]. In investigating the quantity of carotene that must be given to restore a normal rate of growth in rats considerable variations in the response of the animals were encountered. The irregularities were such as to suggest that they arose from imperfect absorption of the pigment from the gut. The examination of the excreta showed that there were adequate grounds for this view, in that a considerable proportion of the pigment could be recovered from the faeces. At the time this observation was first made the basal diet being employed was almost devoid of fat and the carotene was being administered in the form of a solution in ethyl laurate. This suggested that the absence of fat might be the cause of the poor absorption of the pigment, and a series of experiments was planned to test this view.

Three types of basal diet were used. They were compounded as follows:

1. Fat-free diet

Purified caseinogen[1]	22
Pure glucose	73
Salt mixture	5

2. Low fat diet

"Light-white casein" (B.D.H.)	20
Rice starch	71
Salt mixture	5

[1] The caseinogen was purified by two precipitations with acetic acid and thorough washing after each precipitation. After pressing out the excess of water, the granular mass was dried by treatment with absolute alcohol, then with ether and finally in a hot air-oven at 90° for a few minutes. The dried product was ground to a fine powder and extracted with ether in a Soxhlet extractor for 72 hours.

3. Fat-containing diet

"Light-white casein"	20
Rice starch	61
Hardened cottonseed oil ...	10
Salt mixture	5

All the animals received in addition a daily supplement of 0·003 mg. of irradiated ergosterol dissolved in liquid paraffin and a preparation of an alcoholic extract equivalent to 0·5 g. of dried brewer's yeast. When the animals were showing signs of vitamin A deficiency, known amounts of carotene were administered, either in the form of a solution of the pure pigment in ethyl laurate or as palm oil. The excretion of the pigment by the alimentary tract was estimated colorimetrically.

The results are given in Table I.

Table I.

Experiment	Diet	Carotene administered	% carotene recovered from the faeces
1	(1) Fat-free	0·5 mg. in ethyl laurate	84·9
2	,,	,, ,,	86·5
3	,,	,, ,,	91·8
4	,,	0·1 mg. as palm oil (60 mg. of the oil)	40·9
5	(2) Low fat	0·5 mg. in ethyl laurate	91·3
6	,,	,, ,,	85·5
7	,,	0·1 mg. as palm oil (60 mg. of the oil)	38·1
8	,,	,, ,,	38·0
9	,,	,, ,,	38·0
10	,,	,, ,,	40·0
11	,,	0·1 mg. in ethyl laurate	93·7
12	(3) 10 % fat	,, ,,	11·7
13	,,	,, ,,	11·7

On the assumption that the yellow colour of the fats extracted from the faeces was due solely to carotene, these results illustrate in a striking manner how the absorption of the pigment from the intestinal tract may be influenced by the composition of the diet. Moore [1931] has recently reported that the fat extracted from the excreta of rats fed for a long period on a diet containing 15 % of red palm oil contained unchanged carotene in a concentration as high as twelve times that of the ingested oil.

It was, however, necessary to ascertain that the extracted pigment was indeed carotene. For this purpose a more detailed colorimetric and spectroscopic examination was made of the fats extracted from the faeces and the contents of the caecum of rats fed on a fat-free diet supplemented by a high daily dose of 0·5 mg. of carotene dissolved in ethyl laurate.

The examination of the faecal fats showed that the yellow colour was, within experimental error, entirely due to carotene. In the case of the fats extracted from the contents of the caecum, however, results of considerable interest were obtained. Measurement of the yellow colour and of the blue

colour produced with antimony trichloride showed that carotene was not the only chromogenic substance present. The carotene content measured spectroscopically was 1·4 %, while on the basis of the intensity of the 590 $m\mu$ band produced with antimony trichloride the percentage calculated was 2·7 %. Carotene accounted, therefore, for only some 50 % of the blue colour. Spectroscopic examination of the fat in 1 % solution in alcohol in 2 mm. thickness showed a well-defined band at 338 $m\mu$ (Curve *A*, Fig. 1). By referring to the

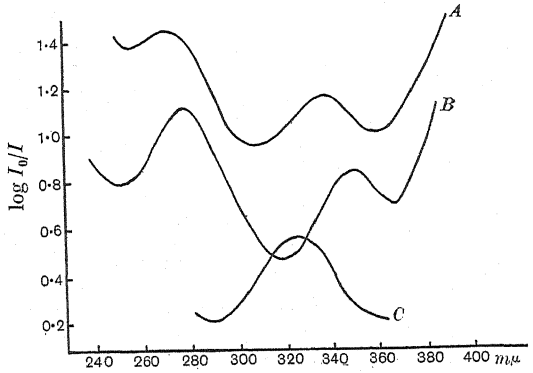


Fig. 1. Spectroscopic examination of the caecum fat. Curve *A*. Caecum fat 1 % solution in alcohol 2 mm. thickness. Curve *B*. Carotene content of the caecum fat calculated theoretically. Curve *C*. By subtracting *B* from *A*; a typical vitamin A curve; maximum absorption about 325 $m\mu$.

absorption curve of pure carotene (Curve *B*), it can be concluded that the fat was exhibiting a band at about 325 $m\mu$ of a similar character to that associated with vitamin A (Curve *C*).

If the substance responsible for this band at 325 $m\mu$ was indeed vitamin A, the question arose as to its origin. The alternative explanations were that it was formed in the intestinal tract, presumably by bacterial action, or that it had been formed in the tissues and excreted into the gut by way of the bile. In order to test the first possibility a series of experiments was made on the influence of intestinal bacteria on carotene under different conditions. Colloidal solutions of carotene were prepared, usually by pouring alcoholic or ethereal solutions of the pigment into water, gum saline or broth, shaking the mixtures well and removing the solvents carefully. The addition of small amounts of cholesterol greatly facilitates the preparation of such suspensions and renders them more stable. Such carotene preparations mixed with peptone broth media were inoculated with material from the caeca of the rats that had been kept for some time on a vitamin A-deficient diet, and the cultures were incubated either aerobically or anaerobically at 37° for periods ranging from 1 to 11 days. After incubation the fat-soluble material was extracted by ether and the dried extract was examined for its yellow colour and for its power to give the blue colour with antimony trichloride. A very large number of cultures of this type were made. The results were very inconsistent when judged by the yellow-blue

ratio, which, as Moore [1930] has pointed out, is about 11 for pure carotene. In general the aerobic cultures showed a reduction in both yellow and blue colours pointing to conversion of carotene into products not giving the antimony trichloride reaction. The anaerobic cultures gave very irregular results. In some cases the carotene was apparently unaffected by the bacterial growth, in others some destruction similar to that in the aerobic tubes occurred, whilst in two cases very definite evidence of the conversion of carotene into a substance giving a much more intense reaction with $SbCl_3$ was obtained. The yellow/blue ratio, which for other cultures showed values ranging between 4 and 11, was 1–1·3.

In one case the change was very striking in that the extracted material had lost most of its yellow colour but gave a much more powerful reaction with $SbCl_3$ than the original pigment. The product appeared to be very unstable, for after some hours the reaction with $SbCl_3$ was much less intense, and by the time the sample was examined spectroscopically by Dr Morton the chromogenic property had disappeared. In spite of many attempts, it has not been found possible to reproduce the conditions that gave this interesting result, and it is uncertain, therefore, whether a formation of vitamin A from carotene was brought about by anaerobic bacteria in these two isolated experiments.

The failure of the large proportion of these experiments to demonstrate the production of vitamin A from carotene by intestinal bacteria diverted attention to the possibility that the conversion normally takes place after absorption.

If it be assumed that the change takes place in the tissues, it must be admitted that there is a large amount of evidence to support the view that the liver is the organ chiefly concerned. Sherman and Boynton [1925] and Kerppola [1930] have shown that the liver of the rat contains much more vitamin A than its other organs, whilst the recent work of Moore [1931] and Capper, McKibbin and Prentice [1931] proves the remarkable concentration of the vitamin in the liver that may follow the administration of carotene. Of no less significance are the observations of Channon [1926] and of Channon and Collison [1928, 1929] on the retention in the liver of the greater part of the unsaponifiable substances (*e.g.* cetyl alcohol, liquid paraffin) absorbed from the intestinal tract.

It appeared to be of importance, therefore, to obtain more precise information regarding the rôle of the liver in the formation of vitamin A from carotene.

In the first place the effect of incubating pulped liver tissues with colloidal suspensions of carotene under a variety of conditions was investigated, but the results proved, not unexpectedly, to be of no value. The next step was to attempt the direct perfusion of the intact liver with suitable carotene solutions. For this purpose healthy young adult cats were fed on a diet of cooked lean horse meat and separated milk which can be regarded as having a very low vitamin A content. By observations of the body weight and general condition of the animals it was thought that from 7 to 12 weeks was probably sufficient to deplete their reserves of vitamin, although rather wide variations were

Table II.

No. of cat	Weight of cat (kg.)	Pre-paration period (wks.)	Time of perfusion (hrs.)	Amount of carotene perfused (mg.)	Volume of perfused fluid (cc.)	Name of tissue	Weight of tissue (g.)	Weight of fat (g.)	Yellow units (10 % dil.)	Blue units (10 % dil.)	Total yellow units	Total blue units	Remarks
6	2·5	7·0	9·75	7·0	100	Liver before perfusion	4·7	0·131	6·5	Trace	128	—	No evidence of vitamin A formation
						Liver after perfusion	67·4	1·837	19·6	0·3	380	6·0	
						Blood	75·2	0·094	7·4	Trace	7	—	
						Gall bladder	1·64	0·076	Trace	0	Trace	0	
8	2·6	7·0	1·25	1·0	12	Liver before perfusion	16·4	0·318	31·4	6·2	300	64	Owing to short time of perfusion the animal may be regarded as a control
						Liver after perfusion	70·2	0·627	20·7	6·8	200	65	
						Gall bladder	2·11	0·020	Trace	0	—	0	
7	3·04	8·0	2·9	5·0	33	Liver before perfusion	6·4	2·18	7·7	29·0	270	1000	Supplies of vitamin A in the liver not exhausted; no evidence of vitamin formation
						Liver after perfusion	87·6	3·254	46·1	29·0	1600	1000	
						Blood	38·5	0·11	3·6	Trace	4	—	
						Gall bladder	1·97	0·16	Trace	Trace	—	—	
12	2·58	11·0	17·75	20·0	96	Liver before perfusion	4·3	0·108	2·6	39·4	42	640	No evidence of vitamin A formation
						Liver after perfusion	62·8	1·508	77·8	34·4	1260	560	
10	2·59	11·0	14·75	20·0	100	Liver before perfusion	3·4	0·055	7·8	380	125	6150	Doubtful evidence of vitamin A formation
						Liver after perfusion	61·7	1·550	63	545	1000	8720	

10 % colour units refer in the case of yellow units to a 10 % solution of the fat in chloroform and examined in a thickness of 1 cm.; and in the case of blue units to those obtained by mixing 0·2 cc. of a 10 % solution of the fat with 2 cc. of antimony trichloride reagent, in a 1 cm. cell, the colours being matched after 30 sec.

Total colour units are obtained by the examination of a solution of the total amount of the fat in a suitable volume of chloroform and multiplying the values so obtained with the total volume.

The red or yellow tints which were inserted to match exactly the yellow and blue colours have been omitted from the Table.

Spectroscopic examinations were made by Dr Morton for the 328 $m\mu$ band and the blue colour developed with antimony trichloride reagent.

encountered. When considered to have reached a condition of depletion the animals were placed under amytal anaesthesia, the spleen was tied off, and a small lobe of the liver was removed to provide control material[1]. A colloidal suspension of carotene in gum saline (usually 10 mg./100 cc.) was then slowly infused into the liver at the rate of approximately 10 cc. an hour, through a branch of the splenic vein. A suitable time after the infusion was completed, the animal was bled, and the remainder of the liver excised for examination. The blood and liver samples were quickly ground up with anhydrous sodium sulphate and the fats thoroughly extracted by ether. In some cases the contents of the gall bladder were also examined. The results of the examination of these fats are shown in Table II.

Cat No. 8 may be regarded as a control, for it died when it had been under experiment only for $1\frac{1}{4}$ hours during which it received 1 mg. of carotene. In none of the other experiments is there any clear evidence from the colorimetric estimations of the formation of vitamin A from carotene in the liver. Unfortunately the only experimental result tending to point in that direction (cat No. 10) is rendered questionable by the uncertainty of the estimations on the control sample of the liver tissue. On the other hand the two most satisfactory experiments (cats Nos. 6 and 12) definitely indicate that there was no conversion of carotene into vitamin A in periods of $9\frac{3}{4}$ and $17\frac{3}{4}$ hours respectively. The increase in the yellow colour of the liver fats after perfusion was not as great as might have been expected, and was at first somewhat naturally attributed to carotene. When, however, certain of the preparations were examined spectroscopically by Dr Morton it was found that the greater part of the yellow colour was due to other substances. This pointed to the pigment having undergone considerable change. But even on the assumption that all the yellow colour of the liver fat was due to carotene, there was a great discrepancy between the amounts found in the liver and that administered in the perfusion. Some of the figures are given in Table III.

Table III.

No. of cat	Time of perfusion (hrs.)	Amount of carotene given (mg.)	Carotene in the liver before perfusion (mg.)	Carotene in the liver after perfusion (mg.)	Increase in carotene (mg.)	Amount of carotene unaccounted for (mg.)
7	2·9	5	0·159	1·145	0·986	4·014
6	9·8	7	0·078	0·171	0·093	6·907
10	14·8	20	0·074	0·656	0·582	19·418
12	17·8	20	0·037	0·777	0·740	19·260

Since little or no carotene was detected in the blood and in view of the spectroscopic evidence that the yellow colour of the liver fat is largely due to substances other than carotene, it seems reasonable to conclude that most of the pigment introduced into the blood supply of the liver had been converted into other substances but not appreciably into vitamin A.

In this connection it is interesting to remember that Palmer [1922, Palmer

[1] The operations on these animals were very kindly performed by Prof. C. L. Evans.

and Kennedy, 1921] has for many years past considered the normal yellow lipochrome pigment of the mammalian liver to be a derived carotenoid. It is also appropriate to recall his repeated warnings against concluding that a pigment is carotene on the basis of solubilities and simple colour reactions. Without confirmation from a spectroscopic examination values obtained for the yellow/blue ratio should be interpreted with caution.

The failure of the perfusion experiments to demonstrate conversion of carotene into vitamin A in the livers of cats led to an attempt being made to ascertain whether the vitamin could be detected in the body a short time after the administration of the pigment by the mouth. Young adult cats were fed on the vitamin A-poor diet of cooked horse meat and separated milk. In view of the incomplete depletion of the livers in some of the former group of animals, the preliminary period for this group was fixed at 16 weeks. During this period there was fairly steady growth for the first two months, but in all cases the weight had declined considerably by the end of the four months. One cat (No. 17) was killed to serve as control, although from previous experience it was appreciated that it was a difficult matter to get adequate control material for this particular experiment. Another cat (No. 14) was deprived of food for 12 hours and then given 100 mg. of carotene dissolved in oil and then emulsified with separated milk. Six hours after the administration it was killed for examination. In a third case (Cat No. 18) two doses of 50 mg. of carotene were similarly given, one 20 hours after the other, and the animal was killed 40 hours after the first administration. The results of the examination of the materials obtained from their tissues are set out in Table IV.

The examination of the cats revealed that even after 40 hours a very small proportion of the carotene had been absorbed. By far the greater part remained in the gut in the form of an intensely pigmented fat. The pigment present in the contents of the alimentary tract was proved by spectroscopic examination to be carotene and no evidence of a formation of vitamin A was obtained.

The increase in the yellow colour of the liver-fats and possibly of the blood-fats also, of cats Nos. 14 and 18, suggested that carotene had been absorbed, and the unchanged pigment was detected spectroscopically in the liver fat of cat No. 18. There was, however, no clear evidence from either the colorimetric or spectroscopic examinations to indicate the formation of vitamin A. The liver fat of cat No. 18 showed an indication of a band at 328 $m\mu$ but it was very slight and might have represented a residual trace of vitamin from the preparatory period.

To extend this line of investigation other cats were given small doses of carotene for long periods. The dose was usually 2 mg. a day, given in the form of an oily emulsion in separated milk for periods of 8–13 weeks. Two control animals received 1 cc. of good cod-liver oil in the form of 2 cc. of a 50 % emulsion. The results are shown in Table V.

The controls grew normally and showed almost complete utilisation of the

Table IV.

(The estimations of colour etc. were made as described in the note to Table II.)

Source of fat	Experiment	Cat No. 17 (control)	Cat No. 14 (killed 6 hrs. after 100 mg. dose of carotene)	Cat No. 18 (killed 40 hrs. after two 50 mg. doses of carotene)
Liver	Weight of the tissue (g.)	74·8	96·6	54·2
	Weight of fat (g.)	5·975	5·178	1·984
	Yellow units (10 % dil.)	2·6	7·2	25·2
	Blue units (10 % dil.)	2·2	0·7	2·6
	Total yellow units	155	373	504
	Total blue units	130	37	52
	Spectroscopic examination	No evidence of carotene or 328 $m\mu$ band	No carotene or 328 $m\mu$ band	Mainly carotene, 328 $m\mu$ band doubtful
Blood	Weight of fat (g.)	0·075	0·292	0·242
	Yellow units (10 % dil.)	8·3	13·7	13·2
	Blue units (10 % dil.)	0	0	0
	Spectroscopic examination	No carotene or vitamin A detected	No carotene or vitamin A detected	No carotene or vitamin A detected
Stomach	Weight of fat (g.)	—	2·254	1·22
	Yellow units (10 % dil.)	—	1,100	170
	Blue units (10 % dil.)	—	33	7
	Total yellow units	—	25,000	2,000
	Total blue units	—	750	80
	Spectroscopic examination	—	Carotene present, but no vitamin A	Carotene present, but no vitamin A
Intestine	Weight of fat (g.)	—	0·918	1·337
	Yellow units (10 % dil.)	—	4,000	240
	Blue units (10 % dil.)	—	92	7
	Total yellow units	—	37,000	1,200
	Total blue units	—	850	90
	Spectroscopic examination	—	Carotene present, but no vitamin A	Carotene present, but no vitamin A
Caecum	Weight of fat (g.)	—	0·024	1·026
	Yellow units (10 % dil.)	—	5,200	6,900
	Blue units (10 % dil.)	—	100	100
	Total yellow units	—	1,250	71,000
	Total blue units	—	25	1,050
	Spectroscopic examination	—	Carotene, present but no vitamin A	Carotene present, but no vitamin A
Gall bladder	Weight of fat (g.)	0·006	0·018	0·017
	Yellow units	Trace	Trace	Trace
	Blue units	0	0	0

vitamin A supplied in the cod-liver oil supplement, as no trace of it could be detected in the faeces. The livers showed considerable storage of vitamin A and there were apparently traces of it present in the blood. These results are in marked contrast to those obtained on examining the cats fed on carotene, which show comparatively small vitamin deposition in the liver. In part this result was due to imperfect absorption, for during the feeding period it was

Table V.

No. of cat	Dose	Tissue	Weight of tissue (g.)	Weight of fat (g.)	Yellow units (10 % dil.)	Blue units (10 % dil.)	Total yellow units	Total blue units
4	1 cc. of cod-liver oil for 8·5 weeks	Liver	76·5	1·834	27·1	435	500	8000
		Blood	78·2	0·22	3·0	3·2	6·6	7·0
		Gall bladder	1·98	0·017	0	0	0	0
3	Ditto	Liver	75·7	1·831	26·3	210	480	3850
		Blood	94·8	0·111	5·4	Trace	6·0	—
		Gall bladder	1·62	0·023	0	0	0	0
1	2 mg. carotene for 8·5 weeks	Liver	67·7	1·734	11·6	4·0	130	70
		Blood	81·0	0·084	5·9	3·5	5·0	3·0
		Gall bladder	1·17	0·015	Trace	Trace	—	—
2	Ditto	Liver	79·7	3·452	21·4	2·5	740	90
		Blood	90·0	0·22	3·2	0	7·0	0
		Gall bladder	1·240	0·021	0	0	0	0

ascertained that the cats were excreting approximately half of the pigment administered. The absorbed portion should, however, have been more than sufficient for the requirements of the animals on the basis of comparison with rats, although the work of Capper, McKibbin and Prentice [1931] suggests that there may be considerable differences between species in respect to the amount of carotene required.

Summary.

1. The absorption of carotene from the intestinal tract may be considerably affected by the composition of the diet. Rats fed on a fat-free diet excreted as much as 90 % of the pigment when it was administered in the form of a solution in ethyl laurate. Absorption was better when the carotene was given in the form of palm oil and was nearly complete when the basal diet contained 10 % of fat. Imperfect absorption was also observed in cats fed on a diet of lean meat and separated milk.

2. Evidence was obtained of the presence of a substance resembling vitamin A in the contents of the caecum of rats fed on a diet deficient in vitamin A but supplemented with carotene.

3. Experiments were made to ascertain whether carotene can be converted into vitamin A by the action of intestinal bacteria. The great majority of these tests gave negative results but in two cases of anaerobic incubation a substance of pale yellow colour, giving a more intense reaction with antimony trichloride than the original pigment, was formed, but it was not found possible to identify it as vitamin A.

4. Attempts to convert carotene into vitamin A by incubation with liver tissue *in vitro* failed.

5. An attempt was made to ascertain whether carotene is converted in the liver into vitamin A by perfusing the organ in cats whose reserves of the vitamin had been depleted by a long period of feeding on a vitamin A-poor diet. No evidence of such conversion was obtained in periods up to $17\frac{3}{4}$ hours.

6. Administration of large doses of carotene by the mouth to cats whose reserves of vitamin A had been depleted did not result in the appearance of

vitamin A in the liver or blood up to 40 hours. A large proportion of the carotene was not absorbed but evidence was obtained to show that the pigment that entered the tissues was converted into other substances.

I wish to express my deep indebtedness to Prof. J. C. Drummond for his kind help and advice throughout the course of this investigation and to Dr R. A. Morton for carrying out the spectroscopic examination of the fats. The expenses of this investigation were defrayed from a grant of the Medical Research Council to Prof. J. C. Drummond.

REFERENCES.

Capper (1930). *Biochem. J.* **24**, 980.
—— McKibbin and Prentice (1931). *Biochem. J.* **25**, 265.
Channon (1926). *Biochem. J.* **20**, 400.
—— and Collison (1928). *Biochem. J.* **22**, 391.
—— —— (1929). *Biochem. J.* **23**, 676.
Drummond, Ahmad and Morton (1930). *J. Soc. Chem. Ind.* **49**, 291T.
Kerppola (1930). *Skand. Arch. Physiol.* **56**, 181.
Moore (1929). *Lancet* ii, 380.
—— (1930). *Biochem. J.* **24**, 692.
—— (1931). *Biochem. J.* **25**, 275.
Palmer (1922). Carotinoids and related pigments. (New York.)
—— and Kennedy (1921). *J. Biol. Chem.* **46**, 559.
Sherman and Boynton (1925). *J. Amer. Chem. Soc.* **47**, 1646.

CXXXIV. THE DISTRIBUTION OF THE VITAMIN B COMPLEX.

II. ROOT VEGETABLES.

By MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

(*Received July 1st, 1931.*)

The following work is a continuation of that already carried out in this laboratory on the estimation of the amount of the antineuritic vitamin B_1 and the antidermatitis vitamin B_2 in naturally-occurring foodstuffs [Aykroyd and Roscoe, 1929; Aykroyd, 1930; Roscoe, 1930].

Since this work was started several constituents of the vitamin B complex other than vitamins B_1 and B_2 have been differentiated by various workers. Vitamin B_3 [Williams and Waterman, 1928] has been shown to be necessary for the growth of pigeons but not for that of rats, on which these experiments have been carried out. Vitamin B_4 has been shown by Reader [1928, 1929, 1930] to be necessary for the continued growth and well-being of rats, and the possibility of its presence or absence having affected the assays of vitamins B_1 and B_2 described in this and former papers is discussed later.

Historical.

The presence of the antineuritic vitamin (B_1) in the potato was established by the early workers with pigeons. Vedder and Clark [1912] obtained partial prevention of polyneuritis with 10 g. of the fresh tuber. McCollum and Kennedy [1916] obtained a slow cure with potato juice and Chick and Hume [1917] with an alcoholic extract of the peelings. An extract of the peeled tuber improved the condition of the birds but did not cure the nervous symptoms.

A number of American workers tested various root vegetables by feeding them to rats as the sole source of the vitamin B complex [McCollum, Simmonds and Parsons, 1918; Denton and Kohman, 1918; Steenbock and Gross, 1919]. None of these tests, however, can be interpreted to show minimal amounts of the roots providing adequate supplies of either vitamin B_1 or B_2, since it must remain uncertain which of the two was acting as limiting factor. Moreover the vitamin-containing substances were fed as percentages of the diet and as will be shown later this method is not capable of quantitative interpretation.

Osborne and Mendel [1919, 1920] investigated the daily dose of roots

[1] Beit Memorial Fellow.

necessary for the normal growth of rats when supplementing a basal diet, the protein of which was an extracted meat residue. It seems probable from control experiments [Osborne and Mendel, 1917] that this residue contained vitamin B_2 but no vitamin B_1, and that their experiments may, therefore, be interpreted as vitamin B_1 estimations. They found that 1·0 g. daily dry weight of either carrot or turnip was needed for good growth, 1·0 g. dry weight of either old or new potato only provided enough vitamin for subnormal growth, 2·0 g. was enough for approximately normal growth. The peel was not found to be a better source of this vitamin than the inside. 2·0 g. of beetroot supported fairly good growth.

Dunham [1921] used the weight maintenance of 4–5 weeks old rats as criterion of "vitamin B" content. Since vitamin B_2 is not necessary for weight maintenance his results may also be interpreted as vitamin B_1 estimations. He found that 0·065 g. of dry potato, 0·07 g. of dry carrot, or 0·105 g. of turnip was needed. The lowness of his figures, as compared with those of Sherman given below, is probably due to the fact that in his experiments coprophagy was not prevented.

Sherman [1926] also quotes figures for the weight maintenance of young rats, but no experimental details are given. He reports that 3·2–3·8 g. fresh (0·32–0·38 g. dry) of carrot and 3·5 g. fresh (0·38 g. dry) of turnip and 3·5 g. fresh (0·7 g. dry) of potato were needed to maintain weight.

Morgan [1924] obtained cures of paralysis due to vitamin B_1 deficiency in rats by daily doses of 1·0 g. of dry radish. The cures and resultant growth were not quite so good as those with rats receiving 0·3 g. of dry brewer's yeast.

In the following paper the vitamin B_1 and vitamin B_2 values of potato, carrot and turnip are reported and compared with those of yeast and the other natural foodstuffs previously investigated. These vegetables were chosen as representative species of the so-called root vegetables, the potato being really an underground stem or tuber. Carrots and turnips are both roots but they differ in that the carrot is highly pigmented and the turnip is colourless. All three are among the most commonly used in ordinary diets.

Methods.

The methods employed were those described in the previous paper [Roscoe, 1930], the growth of young, 40 g., rats being used as criterion. These methods are slight modifications of those of Chick and Roscoe [1928, 1929].

The vitamin B_1 estimations were made using the unpurified caseinogen diet (FL), vitamin B_2 being supplied as autoclaved yeast extract (F5A). The vitamin B_2 estimations were made with the purified caseinogen diet (P2L) with vitamin B_1 as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925].

The relatively large amounts of the roots that it was necessary to administer made it impracticable to give them as daily doses. They were therefore minced and incorporated in the diet, the caseinogen and rice starch of which were varied so as to keep the percentage of protein at 20, the rest of the material being substituted for rice starch. The rats were not, however, allowed to eat more than a given amount of these diets, the remainder of their requirements being made up with FL or P2L.

Diets with the following varying percentages of incorporated vegetables were employed:

10 %	daily ration	2·5 g.	equivalent to a daily dose of	0·25 g.
10 %	,,	5·0 g.	,, ,,	0·5 g.
20 %	,,	5·0 g.	,, ,,	1·0 g.
40 %	,,	5·0 g.	,, ,,	2·0 g.
65 %	,,	6·0 g.	,, ,,	4·0 g.

These figures are for dry weights. All the diets were, however, mixed with 50 % of water. When the roots contained more water than would make up this amount they were reduced before incorporation in the diet by partial drying at 37°. All diets were cooked for 2–3 hours.

THE VITAMIN B_1 CONTENT OF ROOT VEGETABLES.

The results of these experiments are given in Table I. The amounts consumed daily, given in the second column, are expressed as dry weight.

Table I. *Vitamin B_1 content of root vegetables as demonstrated by weight increase of rats during five weeks on a diet, complete, except for vitamin B_1, and containing various amounts of the root vegetables to be tested.*

Material tested	Amount eaten daily, g. dry weight	No. of rats	Increase in weight in 5 weeks (averages, g.)
Carrot	0·25	6	27
	0·5	4	65
	1·0	4	88
Turnip	0·5	4	49
	1·0	5	79
Potato	1·0	4	45
	2·0	4	95

Carrot. This proved a good source of the antineuritic vitamin; rats receiving 0·5 g. gained 65 g. in weight in five weeks, those receiving 1·0 g., 88 g.

Turnip. This was a slightly less good source; rats receiving 0·5 g. gained 49 g. in five weeks, those receiving 1·0 g., 79 g.

Potato. This was a poor source of the vitamin; rats receiving 1·0 g. only increased in weight 45 g. in 5 weeks, those receiving 2·0 g. increased 95 g.

In Table III the amounts of these three root vegetables and of other foodstuffs previously tested needed to give 50–60 g. increase in weight in rats in 5 weeks when given as source of vitamin B_1 are compared and their relative potency is given, yeast being taken as 100.

It will be seen that carrot and turnip are much less potent than yeast in vitamin B_1, approximately equal to the green leafy vegetables and better than egg-yolk or meat. Potato is poorer than any of these.

Potato starch was also tested for its vitamin B_1 content, but the animals would not eat enough to provide a dose of more than 2–3 g. daily and these collapsed with paralysis at the same time as the controls. When receiving a small dose of vitamin B_1 extract, however, the animals on a diet containing potato starch grew better than those on a diet containing rice starch. There would therefore appear to be a small amount of the antineuritic vitamin in potato starch.

THE VITAMIN B_2 CONTENT OF ROOT VEGETABLES.

The results of these experiments are given in Table II. The amounts consumed daily are expressed as the dry weight.

Table II. *Vitamin B_2 content of root vegetables as demonstrated by weight increase of rats during five weeks on a diet, complete, except for vitamin B_2, and containing various percentages of the root vegetables to be tested.*

Material tested	Amount eaten daily, g. dry weight	No. of rats	Increase in weight in 5 weeks (averages, g.)
Carrot	0·5	6	48
	1·0	4	67
	2·0	4	81
Turnip	1·0	4	52
	2·0	4	62
Potato	2·0	4	38
	4·0	4	71

Carrot. This proved the richest of the root vegetables tested in vitamin B_2 as in vitamin B_1; rats receiving 0·5 g. gained 48 g. in 5 weeks, those receiving 1·0 g., 67 g., and those with 2·0 g., 81 g.

Turnip. This was less rich; rats receiving 1·0 g. gained 52 g. and those receiving 2·0 g., 62 g.

Potato. This was a poor source of the vitamin; rats receiving as much as 2·0 g. gained only 38 g. but those with 4·0 g. gained 71 g. during the five weeks of the experiment.

In Table III these vegetables and other foodstuffs are compared with yeast

Table III. *The vitamin B_1 and B_2 content of various foodstuffs compared with that of yeast.*

In columns 1, 2, 4 and 5 are given the minimal amounts needed to promote an increase in weight of 50–60 g. in 5 weeks in young rats of 40 g. weight when given as source of vitamins B_1 and B_2 respectively.

In columns 3 and 6 are shown the relative potencies of the dried foodstuffs when yeast is taken as 100.

		As source of vitamin B_1			As source of vitamin B_2		
	Water content	Weight increase		Potency	Weight increase		Potency
		Wet wt.	Dry wt.	Dry wt.	Wet wt.	Dry wt.	Dry wt.
Material	(%)	1	2	3	4	5	6
Yeast	80	0·25	0·05	100	0·5	0·1	100
Green vegetables	90	3·0–5·0	0·3–0·5	10–17	4·0–6·0	0·4–0·6	17–25
Carrot	90	2·5–5·0	0·25–0·5	10–20	>5·0	>0·5	<20
Turnip	93	>7·0	>0·5	<10	14·3	1·0	10
Egg-yolk	50	<2·0	<1·0	>5	2·0	1·0	10
Meat, steak	75	4·0	1·0	5	2·8	0·7	14
Milk	88	—	—	—	6·0	0·7	14
Potato	78	5·0–10·0	1·0–2·0	<5	10·0–20·0	2·0–4·0	<5

as regards their vitamin B_2 potency, on the basis of the amounts needed of each to promote 50–60 g. increase in weight in 5 weeks.

All are of much lower potency than yeast; carrot has a potency equal to

that of milk, meat and the less good green vegetables, but better than egg-yolk or wheat germ. Turnip is equal to the latter group. Potato is of the same order of strength as the cereals and pulses.

Potato starch was also tested for its vitamin B_2 content. Two rats receiving 60 % potato starch diet and eating such an amount that they received an average of 2·2 and 2·6 g. of starch daily grew better than the controls in the same litter though not better than controls sometimes grow. A very low content of this vitamin is indicated.

Experiments were carried out in which these vegetables were used in the cure of dermatitis arising from a deficiency of vitamin B_2. 0·5 g. of dry carrot, turnip or potato was found to cure symptoms. The minimal amounts needed were not discovered.

The above experiments were carried on for 5 weeks only, but additional experiments were carried out in which the growth to normal adult weight, when the vegetable was the sole source of the vitamin B complex, was investigated.

Three rats, a male and two females, when receiving 2·0–3·0 g. daily of dry carrot followed the normal growth curve. A litter was born to each of the females but only one of the young survived: this one was reared to weaning.

Similarly with 4·0–8·0 g. of potato normal growth curves were obtained and each rat produced a litter. One litter of six was reared to weaning, the other, however, was not fed.

Continued breeding was not investigated.

The effect of the age of the root on the vitamin B content.

A comparison of the vitamin B_1 and vitamin B_2 contents of old carrots which had been stored all winter with those of fresh spring carrots was made. No significant difference was observed. In the vitamin B_1 tests, rats which received 0·25 g. of old carrot increased in weight an average of 29 g. in the 5 weeks of the experiment, those receiving the new carrot 20 g. In the vitamin B_2 tests those receiving 0·5 g. of old carrot increased 42 g. in weight, those with the same addition of new carrot 44 g.

This result recalls that of Osborne and Mendel [1920] who found new potatoes not to be superior in "water-soluble B" to old ones.

The effect of cooking on the availability of the B-vitamins.

In the previous experiments the cooking of the diets in which the vitamin-containing substances were included involved the steaming of the vegetables for 2–3 hours. It was thought possible that this cooking might render them more digestible and so increase the degree of absorption of the B-vitamins from the alimentary canal of the rat. An experiment was therefore performed in which the same quantities of carrot were fed (i) as a percentage of the diet which was cooked for 2–3 hours and (ii) as daily doses of the uncooked vegetable. Vitamin B_1 and vitamin B_2 tests were carried out in this way.

No difference was observable between the two groups. In the vitamin B_1 tests the average increase in weight in 5 weeks with 0·25 g. of cooked carrot was 31 g.; with the same amount of uncooked carrot it was 29 g. In the vitamin B_2 tests with 0·5 g. of cooked carrot there was an increase in weight of 47 g., with the uncooked of 42 g.

The vitamin B_4 content of the vegetables.

Vitamin B_4 deficiency in rats is characterised by failure to grow after about 4 weeks of deprivation and in acute stages by listlessness, a hunched attitude, and inflamed and swollen paws [Reader, 1928, 1929, 1930]. These symptoms have been observed in these experiments among the negative control rats which were receiving vitamin B_2 as autoclaved yeast extract together with a diet containing unpurified caseinogen. It is therefore clear that vitamin B_4 is not present in the basal diet or in the source of vitamin B_2 given in these vitamin B_1 tests. Thus it is possible that these and previous vitamin B_1 estimations may have been affected by a deficiency of vitamin B_4.

Vitamins B_1 and B_4 are, however, very closely related and there is as yet no evidence that they have a different distribution in nature. Moreover in the experiments here reported and in the majority of the previous experiments, normal growth (14–18 g. per week) was obtained with the higher doses at a time when the controls were showing symptoms of vitamin B_4 deficiency; in these experiments therefore vitamin B_4 must have been present in adequate amounts.

A further test was made in which rats on small doses of carrot, after several weeks of subnormal growth, received in addition the preparation of vitamin B_4 described by Reader [1930]. No improvement in growth was observed, showing that the limiting factor was not a deficiency of vitamin B_4.

It may therefore be taken as proved that vitamin B_4 was present in amounts sufficient for normal growth in the vegetables here tested.

In the vitamin B_2 tests vitamin B_4 is present in adequate amounts in the antineuritic yeast concentrate made according to the method of Peters, the charcoal being extracted with acid alcohol.

Discussion.

The vitamin B_1 and vitamin B_2 contents of the root vegetables appear from these results to vary considerably, carrot being a good source of both the vitamins, potato a poor one and turnip slightly poorer than carrot.

The proportion of vitamin B_1 to vitamin B_2 is similar to that found in the majority of the leafy vegetables, twice as much of the material being needed for good growth when given as source of vitamin B_2 as when given as source of vitamin B_1. This is the same proportion as is also found in brewer's yeast.

These results confirm the conclusions of all investigators in showing that carrot is a fairly good source of the B-vitamins and potato a poor one, and more

particularly they agree with those of Osborne and Mendel [1919, 1920] in showing that 1·0 g. of dry carrot and 2·0 g. of potato contain sufficient vitamin B_1 for normal growth of rats. In disagreement with the present work these authors claimed that turnip was as good a source of the antineuritic vitamin as carrot. Beyond this it is difficult to compare the results here obtained with those of previous workers.

The practice of administering the vitamin-containing substance as a percentage of the diet which is freely consumed by the experimental animal, makes the interpretation of many early results very difficult. In vitamin B_1 tests particularly this method is very uncertain in its results.

In such experiments it is possible by careful measurement of the food consumed to assess the daily intake of the vitamin-containing substance, but it is not possible, except by limiting the total intake, to insure that each animal on a given diet shall eat the same amount. Aykroyd has shown (unpublished results) that on a test diet containing a percentage of a substance low in vitamin B_1 some rats may consume only a small amount of food, and as a result do not obtain enough vitamin B_1 to protect them against paralysis, while others eat about twice as much of the diet and thereby acquire enough of the vitamin to increase their weight by 10–13 g. a week. In fact in one case a rat at first grew 10 g. a week and then ceasing to eat well lost weight and developed paralysis.

This fact would seem to disprove, as far as vitamin B_1 is concerned, the theory put forward by Plimmer, Rosedale and Raymond [1927] that the amount of vitamins B_1 and B_2 (no distinction between the two vitamins is made by these authors) needed is directly proportional to the calories consumed. For in experiments such as the above where the vitamin-containing substance is given as a percentage of the diet the proportion of vitamin to calories ingested is constant whatever the amount eaten, yet the deficiency of a rat which develops paralysis must be considered greater than that of one which shows an increase in weight of 10–13 g. a week.

A further fact indicating that the need for either vitamin B_1 or vitamin B_2 is not dependent on the calorie intake is that at the end of the five weeks tests for either of these vitamins the rats were in some cases eating twice as much food as at the beginning, yet although their vitamin supply had not been increased their growth was just as good.

Summary.

1. The vitamin B_1 content of carrot is about one-fifth that of yeast (dry weights), equal to that of the less good leafy vegetables and better than that of egg-yolk or milk. Turnip is not quite so rich in the vitamin, and potato is poor, being only one-fifteenth as rich as yeast.

2. Carrot is one-fifth as good a source of vitamin B_2 as yeast and about equal to milk, meat and the less good leafy vegetables. Turnip is less potent,

having the same potency as egg-yolk and wheat germ. Potato has a low content of this vitamin, comparable with that of the cereals and pulses, but the relatively large amounts of this vegetable eaten may render it an important source of both the B-vitamins in an ordinary diet.

3. Potato starch contains small amounts of both vitamins, B_1 and B_2.

4. New spring carrots contain no more vitamin B_1 or B_2 than do old ones which have been stored all winter.

5. The vitamins B_1 and B_2 in carrots are not rendered more available to the animal consuming them by steaming for two to three hours.

6. Reasons are given indicating the presence of vitamin B_4 in the vegetables tested.

7. The method of testing for the B-vitamins by incorporation of the test material in the basal diet is criticised and it is pointed out that this method is not admissible unless the consumption of the diet is limited.

8. The vitamin B_1 requirements of the rat do not seem to be related to the total amount of food consumed.

My thanks are due to Dr H. Chick for advice and criticism throughout the course of this work, and to Miss D. Graetz for help with the experimental animals.

REFERENCES.

Aykroyd (1930). *Biochem. J.* **24**, 1479.
—— and Roscoe (1929). *Biochem. J.* **23**, 483.
Chick and Hume (1917). *Trans. Soc. Trop. Med. Hyg.* **10**, 141.
—— and Roscoe (1928). *Biochem. J.* **22**, 790.
—— —— (1929). *Biochem. J.* **23**, 498.
Denton and Kohman (1918). *J. Biol. Chem.* **36**, 249.
Dunham (1921). *Mil. Surg.* **48**, 223.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
McCollum and Kennedy (1916). *J. Biol. Chem.* **24**, 491.
—— Simmonds and Parsons (1918). *J. Biol. Chem.* **36**, 197.
Morgan (1924). *Amer. J. Physiol.* **69**, 634.
Osborne and Mendel (1917). *J. Biol. Chem.* **32**, 309.
—— —— (1919). *J. Biol. Chem.* **39**, 29.
—— —— (1920). *J. Biol. Chem.* **41**, 451.
Peters (1924). *Biochem. J.* **18**, 858.
Plimmer, Rosedale and Raymond (1927). *Biochem. J.* **21**, 913.
Reader (1928). *Chem. Ind.* **47**, 1247.
—— (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77.
Roscoe (1930). *Biochem. J.* **24**, 1754.
Sherman (1926). Chemistry of food and nutrition, 3rd edition, p. 406. (New York.)
Steenbock and Gross (1919). *J. Biol. Chem.* **40**, 501.
Vedder and Clark (1912). *Philippine J. Sci.* **7**, B. 423.
Williams and Waterman (1928). *J. Biol. Chem.* **78**, 311.

CXXXV. A DIFFERENTIAL RESISTANCE THERMOMETER FOR BIOLOGICAL USE[1].

By DOUGLAS ROY McCULLAGH and EDWIN MARTIN CASE[2].

From the Biochemical Laboratory, Cambridge.

(*Received September 5th, 1930.*)

The need has arisen for a micro-calorimeter suitable for the measurement of heat-production in biological material dissolved or suspended in a small amount of liquid. With this object in view a differential resistance thermometer of the type used by Watson and Henderson [1928] to measure the heating effects of thorium products has been constructed. The following is a description of an apparatus designed in order to measure the heat produced by about 1 g. of resting anaerobic muscle in about 2 cc. of Ringer's solution. The details of the arrangements could readily be altered and made suitable for many other purposes.

Principle of the method.

The resistance of a metal to an electric current varies as the temperature of the metal is altered. A, B, C and D in Fig. 1 are the resistances of the arms of

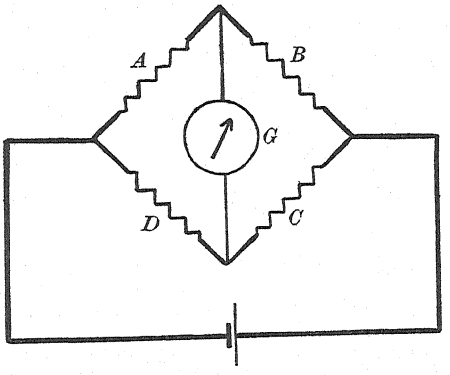


Fig. 1.

a Wheatstone bridge. When the relationship $AC = BD$ holds good, no current passes through the galvanometer G. If now the opposite arms B and D be heated, their resistance will alter and the bridge will no longer be balanced. This change in balance is the basis of the method. It is not measured directly

[1] A brief account of this was given before the Biochemical Society, but a more detailed description seems desirable.

[2] Beit Memorial Research Fellows.

by galvanometer deflection, but the bridge is re-balanced by means of an external variable resistance in parallel with one of the other two arms. The value of this compensating resistance which is necessary to restore the equilibrium of the bridge gives a measure of the heat which has been produced.

Details of arrangement.

In the actual apparatus (Fig. 2) each of the four arms of the Wheatstone bridge consists of No. 47 s.w.g. double silk-covered copper wire, having a resistance of about 200 ohms.

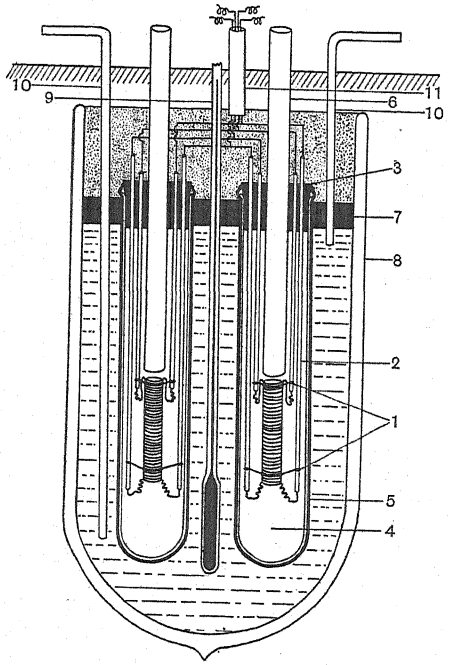


Fig. 2.

Arms A and C are wound on a silver-plated copper tube 7 cm. long and 1 cm. in diameter, with walls 0·15 mm. thick. By means of rubber bands and silk threads (1) this is mounted upon glass tubes (2) which carry the leads from the coils through a rubber stopper (3). This is then placed in a paraffined glass test-tube (4) which is enveloped in a portion of the inner tube of a bicycle tyre (5). Through the rubber stopper a glass tube (6) passes, whose diameter is slightly greater than that of the metal tube.

Arms B and D of the bridge are arranged in exactly the same manner in another similar paraffined tube. Both of the large test-tubes are sealed into a cork (7) which fits into a silvered Dewar flask (8) 5″ in diameter and 10″ deep. Passing through this cork into the interior of the Dewar flask are a high-sensitivity mercury thermometer (9) and two glass tubes (10).

Great care is taken to ensure that all the joints between test-tubes and cork and cork and Dewar flask are completely watertight, and this is especially important with regard to the fitting of the rubber stoppers into the test-tubes. The presence of the merest traces of water inside the tubes is sufficient to render the apparatus useless.

The space between the corks and the rim of the Dewar flask is filled with paraffin wax of melting-point 50°. In this paraffin are embedded the soldered connections completing the Wheatstone bridge. The four leads from the bridge are taken off through a glass tube (11) which is also embedded in the paraffin. The Dewar flask with its contents is then placed in paraffin wax in a cylindrical zinc container, and the whole is immersed in a constant temperature water-bath so that the only portions protruding from the water are the glass tubes (6) by means of which material can be lowered into the metal tubes; the glass tubes (10) by which water of the desired temperature can be introduced into the flask; and the glass tube (11) through which the copper leads pass.

The arrangement of the electrical connections to the four resistances A, B, C and D is shown in Fig. 3. As already indicated, the resistances are soldered together in the form of a Wheatstone bridge. Two leads go to a 2 v. accumulator (Ac); the circuit can be opened or closed by the switch (S_1). One accumulator lead passes through a milliammeter (MA), and the other through a variable resistance (R_1) by means of which the current through the bridge can be regulated accurately. The two galvanometer leads pass through a reversing switch (S_2). One of them also goes through a copper tap key (T) which when closed connects the galvanometer to the bridge circuit, and when open completes the circuit of a galvanometer damping resistance (R_2). Another variable resistance (R_3) is a shunt resistance in parallel with resistance C. The resistances A, B, C and D are made of such magnitude that when a current is passing through the bridge and the shunt resistance is set at about 9000 ohms, no current goes through the galvanometer. This variable resistance (R_3) is capable of accurate adjustment to 0·1 ohm.

The instrument is calibrated by the production of a known amount of heat, for which purpose a small heating coil is constructed and mounted as shown in Fig. 4. The coil (H) is made of cotton-covered constantan wire of 46 s.w.g., and has a total resistance of 32·10 ohms. It is sealed into a small glass bulb (Z), through the capillary neck of which pass leads of No. 47 s.w.g. copper which are soldered to the heating coil. On emerging from the capillary, these leads are in turn soldered to 24 s.w.g. copper wires passing through a rubber bung, by which the weight of the whole contrivance is supported. The capillary and bulb are attached to another rubber stopper which is of such a size as to fit exactly into a small glass tube (V), which in turn slips into the metal tube around which the resistances B and D are wound. The tube (V) and its contents can be lowered through the glass tube (6) in Fig. 2.

A duplicate heating coil *etc.* is made for the other metal tube. The function

of this duplicate coil is merely that of keeping the heat capacity of both halves of the Wheatstone bridge the same, and for this reason it is not necessary that it be constructed with such care. The copper leads from the duplicate are not attached to anything.

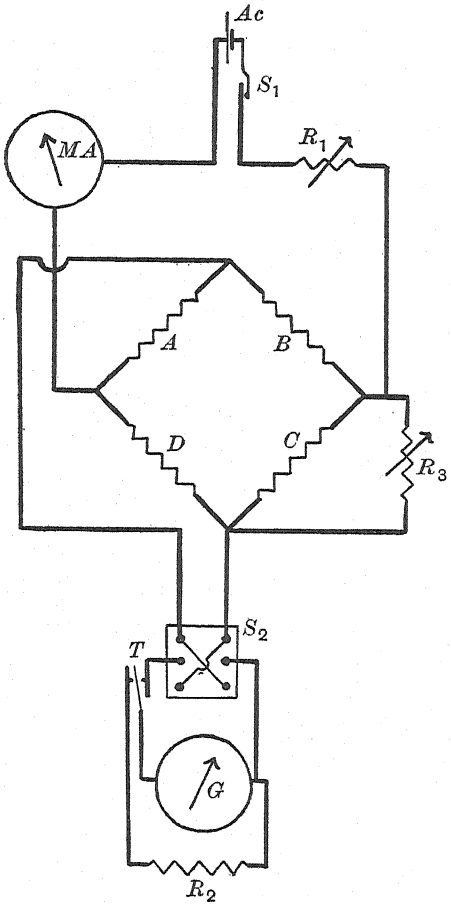


Fig. 3.

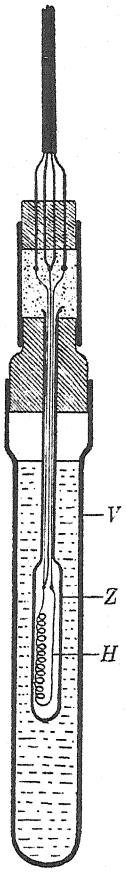


Fig. 4.

The connections to the heating coil are made as shown in Fig. 5. H is the heating coil, and L_1 its leads of 47 S.W.G. copper. R is a constantan resistance equal to the coil resistance, and L_2 are dummy leads of No. 47 S.W.G. copper, whose resistance is the same as that of L_1. These dummy leads in the actual apparatus are enclosed in the bulb and capillary together with the heating coil and its leads. P is a potentiometer, S a copper switch, W a slide-wire, and Ac a 2 v. accumulator. The position of the sliding contact T on the wire W governs the amount of current passing through H.

When points 1 and 2 on the switch are connected to points 3 and 4 respectively a current passes through H, and the amount of this current is known

by measuring the potential difference between the ends of the resistance. The measurement is made on the potentiometer, which in this position of the switch is connected to the heating coil. If points 1 and 2 are connected to points 5 and 6, the potentiometer is no longer connected to the heating coil. The

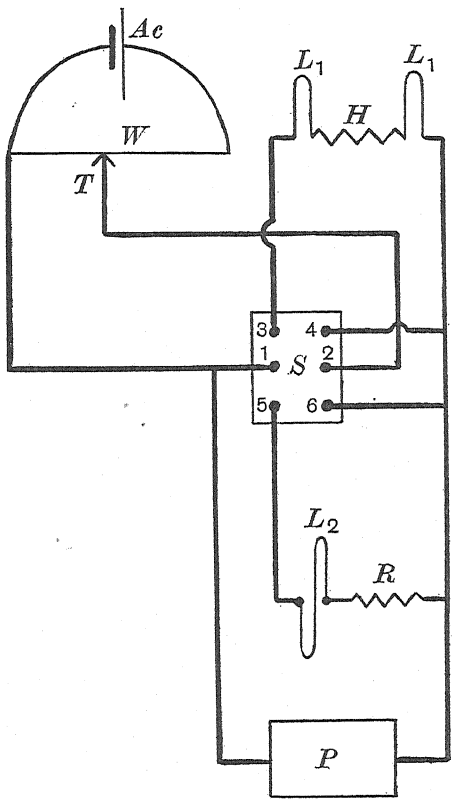


Fig. 5.

current from the slide-wire passes, under these circumstances, through an external resistance R equal to H, and then through the dummy leads L_2; it does not, however, actually pass through the heating element H.

The galvanometer in use is a moving-coil instrument made by the Cambridge Instrument Company. It has an internal resistance of 14·6 ohms, and at 1 m. scale distance gives a deflection of 600 mm. per milliamp. It can be read to 0·2 mm. at a scale distance of 2 m.

Constant temperature water-bath. The bath is essentially the same as that used by Meyerhof. It has been described by Blaschko [1928]. Small alterations have been described by Meyerhof, McCullagh and Schulz [1930]. The temperature of the bath is constant to within 0·002°.

Procedure.

Calibration. The current across the bridge is adjusted to 4 milliamp. and the temperature of the water in the Dewar flask brought to equilibrium with that in the bath by passing water through the glass tubes (10, Fig. 2).

When a large series of experiments is being carried out over a period of several days, the bridge current and the automatic thermoregulator are permitted to run continuously. This results in a constant zero, *i.e.* the amount of resistance in the shunt required to balance the bridge is in this way kept at the same value.

Although the whole system is made of copper, certain unavoidable thermo-electric effects arise. These are eliminated by averaging the readings obtained when the galvanometer current is direct and when it is reversed.

3 cc. of water are placed in each of the two small glass tubes V (see Fig. 4 and text). The heating coil and the duplicate are placed firmly into the tubes, which by means of the leads are then lowered through the glass tubes ((6), Fig. 2) into their positions in proximity to the resistances A and C, and B and D, of the Wheatstone bridge. When the system has attained thermal equilibrium, as indicated by the amount of shunt resistance required to balance the bridge remaining constant, the value of this resistance is noted. This gives the zero point. It is affected very slightly by changes in room temperature; the error due to this is constant and is allowed for. A current is now passed through the heating coil (H, Figs. 4 and 5) and the potential difference between the ends of H is determined.

From the two equations

$$i = E/R \quad \text{and} \quad C = 0{\cdot}239 i^2 Rt,$$

where i = current in ampères, E = potential difference in volts, R = resistance in ohms, t = time in seconds, and C = heat in gram calories during time t, we obtain

$$C = \frac{0{\cdot}239\, E^2 t}{R}.$$

In the case of the apparatus being described the resistance of the heating coil is 32·10 ohms, and we wish to express the heat as gram calories per hour, hence

$$\text{Gram calories per hour} = \frac{0{\cdot}239 \times 3600 \times E^2}{32.10}$$
$$= 26{\cdot}8\, E^2.$$

Thus, if a current is passing through the heating coil and the potentiometer reading is 0·100, the heat production is $26{\cdot}8 \times (0{\cdot}1)^2$, or 0·268 g. cal./hour.

After the heating current has been passed for about 30 minutes the system comes to an equilibrium, as the heat loss is equal to the heat production. Two arms of the bridge are now at a higher temperature than the other two arms and for this reason the shunt resistance will have to be adjusted to a new value in order to bring the galvanometer to zero. In this manner the amount of resistance required in the shunt to balance the bridge can be ascertained for any heat production.

The heat produced by a known weight of muscle can now be measured very rapidly. The glass tube containing the heating coil is withdrawn, and the water is removed and replaced by the muscle, the weight of which should be of the order of 1 g. Ringer's solution is added in such quantity as will bring the heat capacity to its original value. (The specific heat of the muscle is about 0·825, and that of Ringer's solution about 0·995.) The heating coil is replaced, and the whole, after being cooled to a temperature rather below that of the bath, is again lowered into the apparatus. The attainment of the equilibrium condition is hastened by the use of the heating coil until the bridge is obviously approaching its state of balance. When the balance is very nearly reached the switch (*S*, Fig. 5) is put in the position where points (1) and (2) are in contact with points (5) and (6). The sliding contact *T* is then adjusted to the position it would occupy if the amount of heat being produced by the muscle were being produced by the heating coil. This results in the passage of a current through the dummy leads of the coil, of the same magnitude as that which passes through the actual leads during calibration. Thus the heat production in the leads need receive no other consideration.

The heat production of the muscle is known from the amount of resistance necessary in the shunt to balance the bridge after complete equilibrium has been established.

In some cases it is simpler to calibrate the apparatus after the experiment. If the muscle is not required for subsequent chemical measurements it can be killed by placing the small tube containing it in boiling water. The tube and contents are then cooled, dried with ether and replaced in the apparatus. After complete equilibrium has again been reached the changes in resistance which occurred during the experiment are reproduced by passing a suitable current through the heating coil.

Sensitivity. A heat production in 3 cc. of water of 1 cal. per hour results in the shunt resistance having to be changed 145·2 ohms in order to balance the bridge. The bridge can be balanced accurately to 0·1 ohm. It can be shown mathematically that 0·1 ohm. represents a temperature change of about $3° \times 10^{-5}$. By increasing the bridge current or amplifying the galvanometer current the sensitivity could be greatly increased.

Our thanks are due to the Royal Society for a grant to assist in the purchasing of apparatus, and we are grateful to Sir F. G. Hopkins for his encouragement throughout.

We wish also to express our great indebtedness to Messrs H. W. Hall and F. Johnson of this department, whose technical skill was invaluable to us in the construction of the apparatus.

REFERENCES.

Blaschko (1928). Die Methodik der Fermente, Oppenheimer and Pincussen, 689. (Georg Thieme, Leipzig.)
Meyerhof, McCullagh and Schulz (1930). *Pflüger's Arch.* **224**, 230.
Watson and Henderson (1928). *Proc. Roy. Soc. Lond.* A **118**, 318.

CXXXVI. STUDIES ON "NON-IRRITABLE" MUSCLE.

By DOUGLAS ROY McCULLAGH and EDWIN MARTIN CASE[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 2nd, 1931.*)

The condition known as "non-irritability" or "inexcitability," as distinct from fatigue, in amphibian muscle is one to which attention was drawn by Fletcher and Hopkins [1907], though Hermann [1867] had made observations which indicated that the phenomenon had to some extent fallen under his notice. In the course of the classical experiments of Fletcher and Hopkins these authors observed that occasionally the frog muscle that they were using ceased to be irritable, in spite of the absence of rigor and the fact that the lactic acid content was not at its maximum, and that the condition was therefore not one of ordinary fatigue. The nature of the phenomenon was not further studied at the time, and it was not until 14 years later that the work of Foster and Moyle [1921] was published. In their paper it was shown that the condition of non-irritability could readily be produced in frog's muscle by keeping it at a temperature of 0° in an atmosphere of oxygen, air or nitrogen, the time necessary varying according to the gaseous medium employed. Two or three days were sufficient in the case of nitrogen, whereas two or three weeks were required for oxygen, whole limbs being used in both cases. When oxygen was used there was no rigidity at the end of the period, a circumstance which is in conformity with the experiences of Fletcher and Hopkins.

Foster and Moyle then made an investigation of some of the chemical properties of amphibian muscle in the non-irritable state, and they found that heat, toluene, and mechanical injury all have the same effect as in the case of normal muscle; that is to say, all these treatments result in the production of large amounts of lactic acid. An extension of these studies to some of the physical properties led to the conclusion that the loss of irritability is associated with a redistribution of certain ions; perhaps as a result of changes in membrane permeability, or perhaps caused by adsorption on colloidal surfaces. This "ionic" theory was supported by the very important further observation that the loss of irritability was reversible; that by treating the inexcitable muscle with various solutions it was possible to recover a certain degree of irritability. Thomson's [1928] experiments on the internal conductivity of normal and non-irritable muscle, demonstrating as they do a drop

[1] Beit Memorial Research Fellows.

of over 40 % in respect of the latter, furnish additional evidence that a disappearance of free ions is involved in the causation of the phenomenon. Dulière and Horton [1928], pursuing an investigation of non-irritable frog's sartorii, came to the conclusion that the loss of excitability was dependent upon the diffusion of potassium ions from the interior of the fibres to the interspaces between them.

There thus appears to be a fair accumulation of data which support the hypothesis of what Foster and Moyle called the "ionic" origin of non-irritability in muscle. Whether or not this be accepted, however, a further study of the phenomenon appealed to the present authors from the following standpoint. In view of the fact that the muscle can be restored to its original condition by immersion in certain solutions, it is obvious that it is not in any way irreversibly damaged; we have, in the circumstance that we can at will suppress the excitability of a muscle and at will restore it again, a means ready to hand of eliminating certain phases of the activity of the tissue, thereby leaving the way more open for a study of those that remain. The series of metabolic and energy-producing changes in muscle is so complicated that any technique by means of which it is possible to attain even a partial separation of these processes must be a valuable one. Any addition to the array of facts already discovered in connection with non-irritable muscle may therefore be of importance with regard to elucidation of the processes occurring in normal muscle.

The work to be described in the present paper may conveniently be divided into four sections, *viz.* A. The chemical effects of stimulation of non-irritable muscle; B. The thermal effects of stimulation of non-irritable muscle; C. The resting heat-rate of non-irritable muscle; and D. The caloric quotient of non-irritable muscle.

A. The chemical effects of stimulation of non-irritable muscle.

As has been mentioned above, Foster and Moyle [1921] came to the conclusion that there are "no chemical differences" between non-irritable and normal muscle. They stated that "non-irritable muscle formed lactic acid in exactly the same circumstances as fresh irritable muscle," by which they meant that the effect of heating the muscle, treating it with toluene, or subjecting it to mechanical injury was to give rise to the production of lactic acid whether the muscle was normal or non-irritable. Curiously enough, neither these nor any other workers tried the experiment of directly or indirectly stimulating the muscle electrically, in order to see whether any lactic acid was then produced. To do this becomes of importance, when it is considered that the mechanism of irritability is far from being understood. The fact that a non-irritable muscle gives no contraction on stimulation may be due to one of at least two causes, namely that (*a*) the mechanism by which lactic acid is formed from carbohydrate as a consequence of stimulation may be inhibited, or (*b*) lactic acid may still be produced in the ordinary way, but

may be unable to bring about any response in the muscle on account of derangement of the contractile mechanism.

Methods.

(i) *Production of non-irritability in muscles.* A number of skinned hind limb-pairs from frogs were suspended at 0° in a large closed jar, at the bottom of which was a layer of water, out of contact with the limbs, but whose presence was necessary to prevent desiccation of the muscles. Two limb-pairs were connected to silver wires leading outside the jar and were used as tests for irritability, but were not employed in the actual experiments. It was usually found that from 4 days to a week in air produced non-irritability, and that no bacterial or other contamination made its appearance in that time; nor was there any trace of rigidity.

(ii) *Recovery of irritability.* Non-irritable limbs were completely immersed in amphibian Ringer's solution, through which a current of oxygen was continuously bubbled. Excitability began to return as a rule within 20 minutes, as evinced by the response of the muscles to single induction shocks, and after an hour or two's treatment the muscles were in most cases strongly irritable. When the muscles were to be used for subsequent experimentation, it was naturally undesirable to give more shocks than could be helped, for fear of vitiating the results by causing accumulation of lactic acid.

(iii) *Estimation of lactic acid.* The muscles were dissected off the limbs as rapidly as possible on a cold plate and immediately ground in an ice-cooled mortar with sand and 5 cc. of ice-cold 20 % trichloroacetic acid. The pulp was washed into a measuring flask with further trichloroacetic acid and was made up with water to a total volume of 50 cc. Having stood overnight in the ice-chest, the contents of the flask were filtered, neutralised with saturated potassium hydroxide, and interfering carbohydrates removed by the usual copper-lime method. After filtering again, lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927].

(iv) *Estimation of total carbohydrates.* This was performed by the method described by Lohmann [1928].

Results.

Two non-irritable limb-pairs were weighed. All the muscles were then dissected off both right legs and immediately worked up as described above for lactic acid estimation. The limb-pairs were meanwhile weighed again, and the muscles of the remaining legs were stimulated for 10 minutes with continuous induction shocks. These muscles were at once dissected away after the cessation of stimulation and were also treated with trichloroacetic acid. The limb-skeletons were re-weighed; by these successive weighings the amount of muscle taken before and after stimulation was known. Two limb-pairs which had been rendered non-irritable in the same batch as the above two were allowed to regain their irritability in oxygenated Ringer's solution; they then underwent the same sequence of weighing, dissection and stimulation.

The results of the lactic acid estimations are comprised in the first experiment recorded in Table I, which contains also the protocols of other experiments of the same kind made at different times.

Table I.

			Weight of muscle (g.)	Mg. lactic acid per g.	Change in lactic acid content on stimulation
1.	Non-irritable	Right legs	4·350	0·77	—
		Left ,,	4·581	0·76	−0·01
	Recovered	Right ,,	3·118	0·28	—
		Left ,,	2·930	0·49	+0·21
2.	Non-irritable	Right legs	4·59	0·90	—
		Left ,,	4·72	1·00	+0·10
	Recovered	Right ,,	5·85	0·65	—
		Left ,,	5·80	0·89	+0·24
*3.	Non-irritable	Right legs	5·732	0·87	—
		Left ,,	5·540	0·95	+0·08
	Recovered	Right ,,	6·555	0·54	—
		Left ,,	6·391	0·90	+0·36
*4.	Non-irritable	Right legs	4·224	0·65	—
		Left ,,	4·451	0·62	−0·03
	Recovered	Right ,,	5·120	0·38	—
		Left ,,	5·340	0·64	+0·26
5.	Non-irritable	Right legs	4·805	0·88	—
		Left ,,	4·721	0·93	+0·05
	Recovered	Right ,,	5·004	0·58	—
		Left ,,	5·182	0·99	+0·41

* In these experiments the stimulation was direct; in the others it was indirect, *i.e. via* the nerves.

It is noticeable that the lactic acid content of the recovered muscles is always lower than that of the non-irritable ones. This can be ascribed partly to the diffusion of lactic acid in the Ringer's solution, and partly to a certain amount of oxidative resynthesis during recovery.

From these results the conclusion can be drawn that, although in non-irritable muscle the lactic acid-forming mechanism exists and, as Foster and Moyle showed, is able to function under a variety of conditions, nevertheless the ordinary type of stimulation is unable to bring it into play. What has been called the "trigger mechanism" seems to be incapacitated in some way, and the stimulus is unable to initiate the series of reactions leading to the production of lactic acid.

There remains however the possibility that although lactic acid does not appear, carbohydrates are nevertheless disappearing under the influence of the stimulation, but are undergoing some other transformation. To test this point it was necessary to repeat the above experiments, with the difference that instead of lactic acid, total carbohydrates were estimated before and after stimulation.

The results obtained are given in Table II.

Table II.

			Weight of muscle (g.)	Mg. carbohydrate per g.	Change in carbohydrate on stimulation
1.	Non-irritable	Right legs	4·72	1·58	—
		Left ,,	4·66	1·62	+0·04
	Recovered	Right ,,	4·90	2·01	—
		Left ,,	5·25	1·88	−0·13
2.	Non-irritable	Right legs	3·68	1·70	—
		Left ,,	3·51	1·67	−0·03
	Recovered	Right ,,	4·04	2·54	—
		Left ,,	4·19	2·24	−0·30
*3.	Non-irritable	Right legs	4·83	1·76	—
		Left ,,	4·88	1·74	−0·02
	Recovered	Right ,,	3·75	2·63	—
		Left ,,	3·92	2·35	−0·28
4.	Non-irritable	Right legs	3·50	1·43	—
		Left ,,	3·61	1·45	+0·02
	Recovered	Right ,,	4·21	2·10	—
		Left ,,	3·99	1·84	−0·26

* In this experiment direct stimulation was employed.

It is evident from this that in addition to there being no lactic acid production upon stimulation of non-irritable muscle, there is no disappearance of carbohydrate. After recovery in oxygenated Ringer's solution, the muscle is restored to its normal state and stimulation leads to the production of lactic acid and the disappearance of carbohydrate in the usual manner. The conclusion appears justifiable then that the phenomenon of non-irritability has its rise in a failure to act of the so-called "trigger mechanism" and not in a failure of the contractile mechanism to respond. The observation of Foster and Moyle, that stimulation of a non-irritable muscle produces no action current, is worthy of notice in this regard, for there is reason to suppose that the action current is a phenomenon associated with excitation and not with contraction.

B. The thermal effects of stimulation of non-irritable muscle.

For the purposes of studying this question, use was made of the differential resistance thermometer described by McCullagh and Case [1931]. The gastrocnemius of the frog was the muscle employed, and it was dissected out from the non-irritable limb together with the sciatic nerve. The muscle was placed in the tube V [1931, Fig. 4] containing the heating coil, together with distilled water in such amount that the total heat capacity was equal to that of 3 cc. of water. (Ringer's solution could obviously not be used, as it would tend to bring about restoration of the muscle.) The nerve passed through a slot in the stopper of the tube V and lay across two small closely-placed electrodes of silver wire, which in turn were connected to two copper wires which passed up the tube 6 and thence to an induction coil. By this means the muscle could, when desired,

be stimulated *in situ* in the calorimeter without disturbing any part of the apparatus.

The technique of using the apparatus has been fully described in the previous paper and need not be enlarged upon here. After the bridge had attained equilibrium the muscle was stimulated for ten successive periods of 10 seconds, with 10-second intervals, using rapidly-recurring faradic currents from a coil. The balancing resistance values were noted at regular intervals for about an hour, at the end of which period the reading had returned to its original value. The tube containing the muscle was now removed and placed in a beaker of boiling water for 10 minutes, cooled, dried, and put back into the apparatus. When equilibrium was again reached, the stimulation from the coil was repeated in exactly the same manner as before, and fresh readings were taken. The reason for this procedure was that the passage of the stimulating current between the electrodes, although these were not actually enclosed in the tube V, might be expected to give rise to some heat production of a purely physical nature, which would be of the same magnitude whether the muscle were alive or had been killed by exposure to the temperature of boiling water.

Table III.

	Readings of shunt resistance (ohms)	
	Non-irritable muscle	Same muscle after heating
1. Equilibrium	8327·8	8327·9
5 mins. after stimulation	8328·3	8328·2
10 ,, ,,	8328·9	8328·9
15 ,, ,,	8329·2	8329·1
20 ,, ,,	8329·2	8329·2
25 ,, ,,	8329·1	8329·2
30 ,, ,,	8329·0	8329·1
35 ,, ,,	8328·8	8328·8
40 ,, ,,	8328·3	8328·2
45 ,, ,,	8328·1	8328·2
50 ,, ,,	8327·9	8328·0
55 ,, ,,	8327·8	8327·7
60 ,, ,,	8327·9	8327·8
	Constant	Constant
2. Equilibrium	8553·6	8553·4
5 mins. after stimulation	8554·0	8553·9
10 ,, ,,	8554·7	8554·6
15 ,, ,,	8554·9	8550·0
20 ,, ,,	8554·9	8554·8
25 ,, ,,	8554·8	8554·8
30 ,, ,,	8554·6	8554·7
35 ,, ,,	8554·4	8554·5
40 ,, ,,	8554·1	8554·2
45 ,, ,,	8553·9	8554·0
50 ,, ,,	8553·7	8553·7
55 ,, ,,	8553·7	8553·6
60 ,, ,,	8553·7	8553·5
	Constant	Constant

In all the experiments that were made, it was found that the heat production upon stimulation was extremely small, and moreover that it was not

Table II.

			Weight of muscle (g.)	Mg. carbohydrate per g.	Change in carbohydrate on stimulation
1.	Non-irritable	Right legs	4·72	1·58	—
		Left „	4·66	1·62	+0·04
	Recovered	Right „	4·90	2·01	—
		Left „	5·25	1·88	−0·13
2.	Non-irritable	Right legs	3·68	1·70	—
		Left „	3·51	1·67	−0·03
	Recovered	Right „	4·04	2·54	—
		Left „	4·19	2·24	−0·30
*3.	Non-irritable	Right legs	4·83	1·76	—
		Left „	4·88	1·74	−0·02
	Recovered	Right „	3·75	2·63	—
		Left „	3·92	2·35	−0·28
4.	Non-irritable	Right legs	3·50	1·43	—
		Left „	3·61	1·45	+0·02
	Recovered	Right „	4·21	2·10	—
		Left „	3·99	1·84	−0·26

* In this experiment direct stimulation was employed.

It is evident from this that in addition to there being no lactic acid production upon stimulation of non-irritable muscle, there is no disappearance of carbohydrate. After recovery in oxygenated Ringer's solution, the muscle is restored to its normal state and stimulation leads to the production of lactic acid and the disappearance of carbohydrate in the usual manner. The conclusion appears justifiable then that the phenomenon of non-irritability has its rise in a failure to act of the so-called "trigger mechanism" and not in a failure of the contractile mechanism to respond. The observation of Foster and Moyle, that stimulation of a non-irritable muscle produces no action current, is worthy of notice in this regard, for there is reason to suppose that the action current is a phenomenon associated with excitation and not with contraction.

B. The thermal effects of stimulation of non-irritable muscle.

For the purposes of studying this question, use was made of the differential resistance thermometer described by McCullagh and Case [1931]. The gastrocnemius of the frog was the muscle employed, and it was dissected out from the non-irritable limb together with the sciatic nerve. The muscle was placed in the tube V [1931, Fig. 4] containing the heating coil, together with distilled water in such amount that the total heat capacity was equal to that of 3 cc. of water. (Ringer's solution could obviously not be used, as it would tend to bring about restoration of the muscle.) The nerve passed through a slot in the stopper of the tube V and lay across two small closely-placed electrodes of silver wire, which in turn were connected to two copper wires which passed up the tube 6 and thence to an induction coil. By this means the muscle could, when desired,

be stimulated *in situ* in the calorimeter without disturbing any part of the apparatus.

The technique of using the apparatus has been fully described in the previous paper and need not be enlarged upon here. After the bridge had attained equilibrium the muscle was stimulated for ten successive periods of 10 seconds, with 10-second intervals, using rapidly-recurring faradic currents from a coil. The balancing resistance values were noted at regular intervals for about an hour, at the end of which period the reading had returned to its original value. The tube containing the muscle was now removed and placed in a beaker of boiling water for 10 minutes, cooled, dried, and put back into the apparatus. When equilibrium was again reached, the stimulation from the coil was repeated in exactly the same manner as before, and fresh readings were taken. The reason for this procedure was that the passage of the stimulating current between the electrodes, although these were not actually enclosed in the tube V, might be expected to give rise to some heat production of a purely physical nature, which would be of the same magnitude whether the muscle were alive or had been killed by exposure to the temperature of boiling water.

Table III.

	Readings of shunt resistance (ohms)	
	Non-irritable muscle	Same muscle after heating
1. Equilibrium	8327·8	8327·9
5 mins. after stimulation	8328·3	8328·2
10 ,, ,,	8328·9	8328·9
15 ,, ,,	8329·2	8329·1
20 ,, ,,	8329·2	8329·2
25 ,, ,,	8329·1	8329·2
30 ,, ,,	8329·0	8329·1
35 ,, ,,	8328·8	8328·8
40 ,, ,,	8328·3	8328·2
45 ,, ,,	8328·1	8328·2
50 ,, ,,	8327·9	8328·0
55 ,, ,,	8327·8	8327·7
60 ,, ,,	8327·9	8327·8
	Constant	Constant
2. Equilibrium	8553·6	8553·4
5 mins. after stimulation	8554·0	8553·9
10 ,, ,,	8554·7	8554·6
15 ,, ,,	8554·9	8550·0
20 ,, ,,	8554·9	8554·8
25 ,, ,,	8554·8	8554·8
30 ,, ,,	8554·6	8554·7
35 ,, ,,	8554·4	8554·5
40 ,, ,,	8554·1	8554·2
45 ,, ,,	8553·9	8554·0
50 ,, ,,	8553·7	8553·7
55 ,, ,,	8553·7	8553·6
60 ,, ,,	8553·7	8553·5
	Constant	Constant

In all the experiments that were made, it was found that the heat production upon stimulation was extremely small, and moreover that it was not

altered after the muscle had been heated at 100° for 10 minutes. The slight heat changes that did occur, therefore, were to be ascribed to the physical effects of the passage of the current through the electrodes and nerve, and not to any chemical processes taking place in the muscle as a result of stimulation. This finding, that stimulation of non-irritable muscle leads to no heat production, was not unexpected in view of the chemical experiments already reported.

Some typical figures for these thermal experiments are given in Table III.

It is seen that the changes in resistance, which in themselves are very small, run so closely parallel in the cases of the unheated and the heated non-irritable muscle that there can be no doubt that they are not to be attributed to any physiological heat production in the muscle.

C. The resting heat-rate of non-irritable muscle.

A determination of the resting heat-rate of non-irritable muscle under aerobic and anaerobic conditions would be expected to give some indication as to whether the metabolic processes are quantitatively different from those in normal muscle, and as to whether the oxidative reactions are still going on or are suppressed. Several experiments were performed in order to obtain values for the resting heat production, using the differential resistance thermometer and employing the technique described in the previous paper.

The muscle was dissected as carefully as possible from a non-irritable limb, which was weighed before and after dissection. The known weight of muscle was placed in the tube V of the apparatus together with enough distilled water to bring the heat capacity to that of 3 cc. of water. The necessary balancing resistance was determined in the usual way, after which the air in the tube was replaced by nitrogen, this gas being bubbled through the water also for several minutes before re-closing the tube and inserting it in the apparatus. The shunt resistance at equilibrium was again determined. Finally, the tube containing the muscle was placed in a beaker of boiling water for 10 minutes in order to kill the muscle, was dried, cooled, and replaced. A current was passed through the heating coil of such magnitude as to necessitate the same alteration in the shunt resistance as was found necessary during the anaerobic experiment with the live muscle, after which the current was increased so as to obtain the value for the aerobic half of the experiment. The potentiometer readings were taken in both cases, and the heat production calculated from the formula given in the preceding paper.

The figures for the different experiments are set forth in Table IV.

It follows from these figures that the resting anaerobic heat-rate of non-irritable muscle is in the neighbourhood of 0·094 g. cals. per g. of tissue per hour. (This is the mean value of the seven recorded.) The observed variation between 0·080 and 0·117 is by no means larger than is usually encountered in determinations of this nature. The figure 0·094 is in remarkably good agreement with the currently accepted value for the resting anaerobic heat of

Table IV.

No. of experiment ...	1	2	3	4*	5*	6*	7
Weight of muscle (g.)	1·32	1·41	1·52	1·11	1·28	0·72	1·50
Potentiometer reading (aerobic)	0·088	0·102	0·122	0·094	0·097	0·083	0·100
Potentiometer reading (anaerobic)	0·064	0·072	0·081	0·062	0·065	0·051	0·067
Heat production in g. cals. per hour (aerobic)	0·208	0·278	0·398	0·237	0·252	0·185	0·268
Heat production in g. cals. per hour (anaerobic)	0·110	0·139	0·176	0·103	0·113	0·070	0·120
Aerobic heat rate (g. cals. per hour per g.)	0·158	0·197	0·262	0·214	0·197	0·257	0·179
Anaerobic heat rate (g. cals. per hour per g.)	0·083	0·099	0·117	0·093	0·088	0·097	0·080

* In these experiments the muscle was rendered anaerobic by the addition of cyanide instead of the substitution of nitrogen for air. This procedure is seen to make no significant difference to the results.

normal frog's muscle; Hill for instance gives for this the figure 0·092 g. cals. per g. per hour.

Under aerobic conditions, there is not so much constancy in the actual figures obtained, varying as they do between 0·158 and 0·262 g. cals. per hour, with a mean value of 0·211. But the fact that this is more than twice as great as the anaerobic figure, and that in every instance the imposition of anaerobic conditions leads to a substantial fall in the resting heat-rate, furnishes evidence that in non-irritable muscle the oxidative mechanisms are not impaired.

Before the construction of the micro-calorimeter was completed, preliminary experiments along the same lines had been performed, using the Dewar flask technique which is described by Blaschko [1928] and employing large quantities of non-irritable muscle of the order of 20–30 g. The results obtained by this method, which are not incorporated in the present paper, were entirely in agreement with those here reported, in that the anaerobic heat-rate of non-irritable muscle was found to be the same as that of normal muscle, and that the aerobic heat-rate was usually about twice as large as the anaerobic. It was usually found that when this procedure was employed, the actual figures were if anything slightly lower than those yielded by the differential resistance thermometer.

D. The caloric quotient of non-irritable muscle.

The term "caloric quotient" is defined as the heat production (in g. cals.) associated with the anaerobic appearance of 1 g. of lactic acid. It is usually assumed to have a fairly constant value, in the neighbourhood of 390, and recent determinations under conditions of resting anaerobiosis and slight anaerobic fatigue by Meyerhof, McCullagh and Schulz [1930] did not result in any material alteration of this figure. Nevertheless, in the course of several unpublished experiments by one of us (D. R. McC.) it became apparent that this constancy was not invariably to be met with, and a number of instances were encountered in which the value obtained for the caloric quotient was very definitely higher than the accepted figure, although so far as was known

the muscle was in a perfectly normal condition. The fact that from time to time these high caloric quotients were obtained is of great interest from a biochemical standpoint, since the supposed constancy of this ratio is a weighty factor in the evidence for the belief that the only exothermic reaction of quantitative importance taking place in muscle is the transformation of carbohydrate to lactic acid. In cases where an abnormally high caloric quotient is observed, it is clear that there must be occurring other processes which are productive of heat and which do not culminate in the appearance of lactic acid.

The first experiments which were made with the object of studying the caloric quotient of non-irritable muscle were carried out by means of the Dewar flask calorimetric method, and the high figure obtained led us to repeat the observations, using the resistance thermometer, with a view to ascertaining whether the values for the caloric quotient were consistently high when the muscle was non-irritable. As will appear, this was found to be the case, and in no instance was a value lower than 600 obtained.

Technique.

It is desirable in experiments of this kind to avoid, so far as is possible, any injury to the muscle, as such injury leads to exaggerated lactic acid formation. It was customary therefore to use in each experiment the gastrocnemius muscles of two frogs; these muscles can be rapidly removed without damage to any of their fibres. After weighing, the gastrocnemii of the two right legs were placed in the tube V, together with the appropriate amount of water, while those of the left legs were placed in a similar tube with the same amount of water and were treated in an identical manner until the heat measurements were begun. The tubes were made anaerobic by the addition of $M/500$ cyanide and filling with nitrogen. Two or three hours were usually allowed for the apparatus to come to equilibrium, as it was found by experience that for the first hour or two after immersing muscles in water the heat production sometimes tended to be high and irregular, perhaps as a consequence of osmotic processes or perhaps on account of the utilisation of remaining traces of oxygen. At the time of commencing the readings, the tube containing the control muscles, which had hitherto been immersed in the bath, was rapidly cooled in a freezing mixture and its contents (muscle + water) were ground with the addition of a little sand and enough ice-cold 20 % trichloroacetic acid to bring the total volume to 10 cc. Lactic acid estimations were performed upon this, and thus the value for the commencement of the "heat-period" was known. (The assumption made here, that the lactic acid content of corresponding muscles from opposite limbs is identical, is invariably found to be a justifiable one.)

The experiments were allowed to continue for 20 to 24 hours. Preliminary tests had shown that at the end of such a period the muscles were in the same condition as when they were put into the apparatus; that is to say, normal

muscles remained irritable, and non-irritable ones remained non-irritable, but could be restored to irritability in oxygenated Ringer. Bacterial contamination was never encountered under these conditions.

At the expiration of the period, the contents of the calorimeter tube were treated exactly as the control muscles had been, and lactic acid estimations were performed. The apparatus was then calibrated in the usual manner, using 3 cc. of water in the tube V and passing a current through the heating coil.

Table V gives the results of a number of determinations made with normal gastrocnemii; a mean value of 375 is obtained for the caloric quotient. In Table VI are recorded similar experiments in which non-irritable muscle was substituted for normal muscle, and it will be seen that, while the values are considerably more variable, the average figure is much higher, *viz.* 740.

Table V. *Normal muscle.*

No. of experiment ...	1	2	3	4	5
Weight of muscle (g.)	1·20	1·38	1·09	1·23	1·06
Duration of experiment (hrs.)	24	20	20	24	20
Lactic acid before experiment (mg.)	3·9	3·5	4·3	4·5	3·2
Lactic acid after experiment (mg.)	10·9	10·3	9·6	12·4	8·5
Increase in lactic acid—*A* (mg.)	7·0	6·8	5·3	7·9	5·3
Potentiometer reading (volts)	0·063	0·070	0·059	0·071	0·060
Heat produced per hour (g. cals.)	0·107	0·131	0·093	0·135	0·096
Total heat produced—*B* (g. cals.)	2·57	2·62	1·86	3·24	1·92
Caloric quotient ($B/A \times 1000$)	367	385	351	410	362

Mean value of caloric quotient—375.

Table VI. *Non-irritable muscle.*

No. of experiment ...	1	2	3	4	5	6
Weight of muscle (g.)	1·31	1·02	1·22	1·05	1·28	1·17
Duration of experiment (hrs.)	20	22	22	20	20	24
Lactic acid before experiment (mg.)	4·4	3·0	3·7	3·9	4·8	3·0
Lactic acid after experiment (mg.)	7·5	5·1	8·2	6·5	8·8	6·2
Increase in lactic acid—*A* (mg.)	3·1	2·1	4·5	2·6	4·0	3·2
Potentiometer reading (volts)	0·067	0·056	0·068	0·060	0·069	0·059
Heat produced per hour (g. cals.)	0·120	0·084	0·124	0·096	0·128	0·093
Total heat produced—*B* (g. cals.)	2·40	1·85	2·73	1·92	2·56	2·23
Caloric quotient ($B/A \times 1000$)	774	881	607	739	640	697

Mean value of caloric quotient—740.

Since the resting anaerobic heat-rate is the same as in normal muscle, one is compelled to adopt the supposition that in non-irritable muscle the observed heat production is a result of changes other than lactic acid formation; changes which, owing to the suppression of the latter, assume an enhanced quantitative importance. It is not our intention at this stage to put forward any suggestions as to the nature of these processes. In view of these results it appears however that the value is considerably lessened of the supposed "constancy" of the caloric quotient and of the resting heat-rate as bases for the argument that lactic acid production is the main exothermic reaction taking place in muscle. Since the appearance of the work of Lundsgaard [1930] this argument has indeed become increasingly untenable, and there seems to be no escape

from the conclusion that the explanations, hitherto accepted as adequate, for the various thermal relations in muscular tissue will have to be considerably revised. Hartree, for instance [1931], has shown that poisoning by iodoacetic acid, while preventing lactic acid formation, has nevertheless no effect upon the heat distribution during contraction, and the same result has also been arrived at by Fischer [1931]. This may be compared with the fact reported in the present paper, that the resting heat-rate of non-irritable muscle is normal although less lactic acid is being formed. At the same time, the interference can scarcely be of the same, or even similar nature in the two cases, since stimulation of non-irritable muscle does not lead even to carbohydrate breakdown.

SUMMARY.

1. Upon electrical stimulation of "non-irritable" frog muscle, no lactic acid is formed and no carbohydrate disappears.
2. There is no heat production upon stimulation of non-irritable muscle.
3. The resting anaerobic heat-rate of non-irritable muscle is normal.
4. The oxidative mechanisms are intact.
5. The caloric quotient of non-irritable muscle is much higher than that of normal muscle, being of the order of 740 instead of 375.

Our thanks are due to Sir F. G. Hopkins for his continual interest in this research.

REFERENCES.

Blaschko (1928). Die Methodik der Fermente, Oppenheimer and Pincussen, 691. (Georg Thieme, Leipzig.)
Dulière and Horton (1928). *J. Physiol.* **67**, 153.
Fischer (1931). *Pflüger's Arch.* **226**, 500.
Fletcher and Hopkins (1907). *J. Physiol.* **35**, 247.
Foster and Moyle (1921). *Biochem. J.* **15**, 334.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Hartree (1931). *J. Physiol.* **72**, 1.
Hermann (1867). Untersuchungen über den Stoffwechsel der Muskeln.
Lohmann (1928). Die Methodik der Fermente, Oppenheimer and Pincussen, 1242. (Georg Thieme, Leipzig.)
Lundsgaard (1930). *Biochem. Z.* **217**, 162.
McCullagh and Case (1931). *Biochem. J.* **25**, 1213.
Meyerhof, McCullagh and Schulz (1930). *Pflüger's Arch.* **224**, 230.
Thomson (1928). *J. Physiol.* **65**, 214.

CXXXVII. THE ERLENMEYER SYNTHESIS OF AMINO-ACIDS.

By JAMES LAMB and WILLIAM ROBSON.

From the Department of Physiology, University of London, King's College, London.

(*Received July 2nd, 1931.*)

Of the many methods available for the synthesis of α-amino-acids, that originated by Erlenmeyer [1893] has been most generally adopted by biochemists working in this field. For example, in more recent years, Ellinger and Flamand [1907], by its use, synthesised tryptophan, Pyman [1916] histidine, Barger and Ewins [1917] *Pr*-2-methyltryptophan, and Harington and Barger [1927], with a modified procedure, 3:5-diiodothyronine, the immediate precursor in the synthesis of thyroxine.

The main reasons for this choice are, firstly, that the starting materials are more or less readily available, and, secondly, that the benzoyl derivative of the amino-acid, obtainable at one stage of the synthesis, lends itself, as Fischer [1900] showed, to resolution into its optically active isomerides.

Nevertheless, the scheme, as Erlenmeyer himself pointed out, has certain weaknesses and these have been encountered repeatedly by later workers, *e.g.* Ellinger and Flamand, Pyman, Barger and Ewins.

The method as elaborated by Erlenmeyer consisted of four stages, *viz.*: (i) the formation of an oxazolone by the condensation of an aldehyde and hippuric acid; (ii) the hydrolysis of the oxazolone to an α-benzoylamino-acrylic acid; (iii) the reduction of the latter compound to the α-benzoylamino-propionic acid, and finally (iv) the hydrolysis of this compound to the α-amino-acid. Of these, stages (i) and (ii) proceed smoothly and with satisfactory yields. The same, however, cannot be said of stages (iii) and (iv).

The reduction as carried out by Erlenmeyer with the calculated quantity of sodium amalgam yielded only some 10–15 % of the theoretical amount of the saturated acid. This procedure was modified by Fischer who, using much larger quantities of sodium amalgam, obtained 80 % and 65 % yields respectively of benzoylphenylalanine and *N*-benzoyltyrosine. In other cases, however, the yields were persistently poor and moreover, the method has a further disadvantage. When the reduction has been completed, the unreduced acid remaining must invariably first be destroyed before the reduced compound can be isolated in a pure condition.

With regard to stage (iv), the hydrolysis, as carried out by these workers, takes a considerable time to accomplish and the yield of amino-acid is often

unsatisfactory. Thus Erlenmeyer found that benzoylphenylalanine, even after treatment in a sealed tube with concentrated hydrochloric acid for 16 hours at 140°, did not yield phenylalanine quantitatively, while Pyman, using Fischer's modification, reported that benzoylhistidine boiled with 60 times its weight of 20 % hydrochloric acid for 4 hours gave 70 % of the theoretical yield of histidine.

Harington and Barger [1927], in their synthesis of thyroxine on the lines of Erlenmeyer's method, were beset with yet another difficulty. To avoid removal of iodine by the alkaline reducing agent, they substituted for Erlenmeyer's sodium amalgam constant-boiling hydriodic acid and red phosphorus. On subjecting their benzoylaminoacrylic acid to this reducing reagent, they obtained, not the benzoylaminopropionic acid, but the α-amino-acid itself. Later Harington and McCartney [1927], modifying slightly this procedure by the introduction of acetic anhydride as a solvent, synthesised phenylalanine and tyrosine from the corresponding α-benzoylaminoacrylic acids or ethyl esters, whichever proved the more suitable.

Following the publication of these results of Harington and McCartney, Hoffmann-La Roche [1928, 1929] took out patents for the direct synthesis of thyroxine from the oxazolone, the reagent used being essentially that of Harington and McCartney. Apparently the oxazolone, like the benzoylaminoacrylic acid gives satisfactory yields of the amino-acid. Thus Erlenmeyer's original synthesis of an optically inactive α-amino-acid from the aldehyde had been reduced to a three-stage process [Harington and Barger, 1927], and to a two-stage process [Hoffmann-La Roche, 1928, 1929].

Nevertheless it will be seen that both these modifications, otherwise highly successful, suffer from one grave disadvantage. Reference has already been made to Fischer's use of the α-benzoylamino-acid for the purpose of resolution. Yet this is the very stage which is eliminated by the use of the acetic anhydride-hydriodic acid reagent, and thus we find Harington, in order to resolve his synthetic thyroxine (stage iv), under the necessity of doubling back to stage (iii) (he made formyldiiodothyronine), resolving and then hydrolysing the optically active isomerides into the respective amino-acids. It is apparent, therefore, that if the method of Harington and Barger could be so modified that the reduction took place without the simultaneous hydrolysis of the reduced compound, such a procedure would be a distinct advance.

Requiring certain amino-acid derivatives recently for metabolism experiments, and obtaining unsatisfactory yields with the sodium amalgam method of reduction, we turned our attention to the acetic anhydride-hydriodic acid method of Harington and McCartney. It occurred to us, in studying the method as described by these workers, that it might be modified in at least two directions, *e.g.*

(i) by variation of the amount of the hydriodic acid used, and

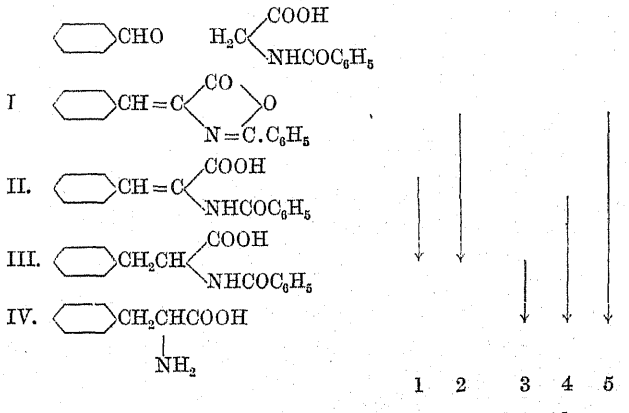
(ii) by the substitution of glacial acetic acid for acetic anhydride as the solvent.

Experimentation on these lines soon indicated that drastic reduction of the amount of hydriodic acid used led to the reduction of the unsaturated compound taking place without the loss of the benzoyl radical from the amino-group. For example, α-benzoylaminocinnamic acid gave benzoylphenylalanine, the product being obtained in excellent yield and in an easily purified form. The reaction was carried on for one hour, although it is probably complete in a shorter period of time. Treated with similar quantities of hydriodic acid, the oxazolone also yields the benzoylamino-acid, although, from results obtained, it would appear that the yield in this case is not quite so satisfactory as that obtained by the reduction of the acrylic acid.

From this point we were led to try the effect of increasing the amount of hydriodic acid, while still retaining acetic acid as the general solvent. It was now found that, should the hydriodic acid be present in sufficient quantity, the reduction was also accompanied by the removal of the benzoyl group, with the consequent production of the α-amino-acid. Thus α-benzoylamino-cinnamic acid under these conditions yields phenylalanine. Here again the yields are good and the product is easily purified. The reaction is complete in from 60–90 minutes, a period which, however, must be considerably lengthened if the hydriodic acid is required to effect the removal of other groups, *e.g.* methyl, as in the synthesis of tyrosine. With similar quantities of hydriodic acid, the oxazolone can be made to yield the α-amino-acid, whilst it also follows that the same method will remove the benzoyl group from the benzoyl-amino-acid.

It will thus be seen that the reaction can be so controlled that the one weakness of Harington and McCartney's procedure is eliminated. Moreover, the reagents involved are such that the synthesis can be carried out at a considerably reduced cost.

The results obtained may be summarised thus:



Reactions 1 and 2—by the use of minimum quantities of hydriodic acid.
Reactions 3, 4 and 5—by the use of much larger quantities of hydriodic acid.

Experimental.

Phenylalanine.

Benzoylphenylalanine. (*a*) From α-benzoylaminocinnamic acid. 10 g. of α-benzoylaminocinnamic acid, prepared according to the directions of Erlenmeyer [1893], were dissolved in 40 cc. of glacial acetic acid A.R., 1·6 cc. of hydriodic acid (sp. gr. 1·7) and 3 g. red phosphorus were added to the solution, and the resulting mixture was boiled under a reflux condenser for one hour. The hot solution was filtered through a glass filter, the separated phosphorus being afterwards washed with hot glacial acetic acid. The combined filtrate and washings were then evaporated to dryness *in vacuo*. Water was added and the evaporation to dryness repeated. The residue was taken up in the minimum quantity of hot alcohol, the solution boiled with a little charcoal and filtered. To the filtrate hot water was added until the solution became cloudy when it was set aside overnight. Practically colourless benzoylphenylalanine separated. A further crystallisation produced a product which melted at 184–5° (Fischer gives M.P. 186°). The yield was 82 % of the theory. (Found: N, 5·2. Calc.: N, 5·2 %.)

An alternative procedure, which, however, does not give quite such a good yield, is to pour the hot reaction mixture, after the removal of the red phosphorus, into a large excess of water. The crude benzoylphenylalanine which separates is purified as described above.

(*b*) From 4-benzylidene-2-phenyl-5-oxazolone. 10 g. of this oxazolone [Erlenmeyer, 1893] were boiled under a reflux condenser with a mixture of 70 cc. glacial acetic acid A.R., 1·6 cc. constant-boiling hydriodic acid and 3 g. red phosphorus for one hour. The reaction mixture was filtered hot and worked up in the manner already detailed. The benzoylphenylalanine, obtained in the first crystallisation from dilute alcohol, melted at 181°. The yield did not equal that obtained from the cinnamic acid, being only 72 % of the theoretical.

Phenylalanine. (*a*) From benzoylphenylalanine. 10 g. of benzoylphenylalanine, prepared by either of the above methods, were dissolved in 40 cc. glacial acetic acid A.R., and to the solution were added 40 cc. of hydriodic acid (sp. gr. 1·7) and a little red phosphorus, the resulting mixture being refluxed for 90 minutes. At the end of this time the hot mixture was filtered, the separated phosphorus washed with hot acetic acid, and the combined filtrate and washings were evaporated to dryness *in vacuo*. Water was added to the residue and the evaporation repeated. The residue was then dissolved in the minimum quantity of boiling water, the solution cooled under the tap, and extracted several times with ether to remove the benzoic acid and any unchanged benzoylphenylalanine. The aqueous solution was then concentrated to about 50 cc. On making it neutral to litmus by the addition of ammonia, the solution began to deposit almost colourless phenylalanine. After standing overnight, the phenylalanine was separated and recrystallised from boiling water. It

was thus obtained in the form of colourless plates. The yield of the analytically pure amino-acid was 5·3 g. or 80 % of theory.

(*b*) From α-benzoylaminocinnamic acid. 10 g. of α-benzoylaminocinnamic acid, 40 cc. of glacial acetic acid A.R., 40 cc. of constant-boiling hydriodic acid and 3 g. of red phosphorus were boiled together under a reflux condenser for 75 minutes. The coloured mixture which resulted was worked up in a precisely similar manner to that obtained in the preparation of phenylalanine from its benzoyl derivative. The yield was 82 % of the theoretical amount, and the product melted at 263–5° (Erlenmeyer gives 263°).

(*c*) From 4-benzylidene-2-phenyl-5-oxazolone. 10 g. of the oxazolone dissolved in 70 cc. of glacial acetic acid A.R. were boiled together with 50 cc. of constant-boiling hydriodic acid and 3 g. red phosphorus for 90 minutes. The subsequent treatment of the reaction mixture followed closely that already described. The yield of phenylalanine obtained amounted to some 68 % of that demanded by theory.

Tyrosine. 4-(p-*Methoxybenzylidene*)*-2-phenyl-5-oxazolone* was synthesised from anisaldehyde and hippuric acid in the usual way. 24 cc. of anisaldehyde, 60 g. of acetic anhydride, 40 g. of hippuric acid and 8 g. of freshly fused sodium acetate were well mixed in a conical flask and heated on the boiling water-bath for half an hour. The solid gradually went into solution and after 20 minutes the oxazolone began to separate; finally the whole became solid. The crude oxazolone was rubbed up with some 300 cc. of hot water and rapidly filtered, a process which was repeated twice. It was then dried in the steam-oven and finally crystallised from benzene, from which it separated in long needles. The product so obtained melted at 161–2°. It weighed 35 g. (62 % of theory). (Found: C, 72·7; H, 4·63; N, 4·8 %. Calc. for $C_{17}H_{13}O_3N$; C, 73·1; H, 4·67; N, 5·04 %.)

*α-Benzoylamino-*p-*methoxycinnamic acid.* The product of the last experiment was dissolved in the minimum quantity of hot alcohol, and to the solution was added 1½ times the theoretical equivalent of sodium hydroxide in the form of a 2 % aqueous solution. The mixture was kept hot and stirred on the boiling water-bath for 15 minutes. It was then cooled and neutralised (Congo paper) with dilute sulphuric acid. The almost colourless solid which separated was sucked as dry as possible on the Büchner funnel, washed several times with distilled water and finally crystallised from dilute alcohol. The yield was quantitative. The melting-point of the product was 230°. (Found: N, 4·6. Calc.: N, 4·7 %.)

*Benzoyl-*p-*methoxyphenylalanine.* (*a*) From α-benzoylamino-*p*-methoxycinnamic acid. A mixture of 10 g. of this acid, dissolved in 35 cc. of glacial acetic acid A.R., 1·4 cc. constant-boiling hydriodic acid and 2·5 g. of red phosphorus was boiled for one hour. The reaction mixture was filtered, and the separated phosphorus washed with hot acetic acid. The combined filtrate and washings were evaporated to dryness *in vacuo*. Water was added and the evaporation repeated. The residue was extracted several times with small

volumes of 10–15 % acetic acid, in which the tar present in the residue was insoluble. The filtrates were combined, boiled with a little charcoal, and filtered. Concentration *in vacuo* of the colourless solution so obtained led to the separation of the desired product. The yield was 65 % of the theory. The melting-point of the product was 173°, and this could not be raised by further recrystallisation from dilute acetic acid or alcohol. (Found: C, 68·1; H, 5·5; N, 4·62 %. Calc. for $C_{17}H_{17}O_4N$; C, 68·2; H, 5·7; N, 4·62 %.)

(*b*) From 4-(*p*-methoxybenzylidene)-2-phenyl-5-oxazolone. The method of preparation of the benzoyl-*p*-methoxyphenylalanine from the oxazolone was exactly similar to that in which the starting-point is the cinnamic acid, with the exception that the amount of glacial acetic acid employed was increased from 35 to 60 cc. The yield in this case was 57 % of the theory, while the product, after the first crystallisation, melted at 172°.

Tyrosine. From (*a*) benzoyl-*p*-methoxyphenylalanine; (*b*) α-benzoylamino-*p*-methoxycinnamic acid; (*c*) 4-(*p*-methoxybenzylidene)-2-phenyl-5-oxazolone.

The preparation of tyrosine from any one of these compounds followed closely that already described for the preparation of phenylalanine from its corresponding derivative. In order to effect the hydrolysis of the methoxy-group the time of reaction was increased from $1\frac{1}{2}$ hours to $3\frac{1}{2}$ hours. It was found best to treat the starting material with 10 times its weight of a mixture of equal volumes of glacial acetic acid A.R. and hydriodic acid (sp. gr. 1·7) together with 2–3 g. of red phosphorus. The yields obtained were (*a*) 75 %, (*b*) 63 % and (*c*) 55 % of those demanded by theory. The specimen of tyrosine prepared from benzoyl-*p*-methoxyphenylalanine darkened at 300° and melted in the neighbourhood of 316°. (Found: N, 7·7. Calc.: N, 7·75 %.)

One of us (W. R.) desires to take this opportunity to acknowledge his indebtedness to the Medical Research Council for a part-time grant.

REFERENCES.

Barger and Ewins (1917). *Biochem. J.* **11**, 58.
Erlenmeyer (1893). *Liebig's Ann.* **275**, 1.
Ellinger and Flamand (1907). *Ber. deutsch. chem. Ges.* **40**, 3029.
Fischer (1900). *Ber. deutsch. chem. Ges.* **32**, 3638.
Harington and Barger (1927). *Biochem. J.* **21**, 169.
—— and McCartney (1927). *Biochem. J.* **21**, 852.
Hoffmann-La Roche (1928). D.R.P. 448838 v. 7. 9. 1928.
—— (1929). E.P. 318582 v. 4. 3. 1929.
Pyman (1916). *J. Chem. Soc.* **109**, 186.

CXXXVIII. A PRELIMINARY NOTE ON THE INORGANIC SULPHATE CONTENT OF THE BLOOD WITH A METHOD FOR ITS DETERMINATION.

By DAVID PATON CUTHBERTSON
and SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received July 2nd, 1931.*)

The methods evolved for the determination of the inorganic sulphate content of the blood may be conveniently summarised as follows: (1) nephelometric, Denis [1921]; (2) volumetric, White [1923]; (3) gravimetric, Loeb and Benedict [1927]; and (4) colorimetric, Yoshimatsu [1926] and Hubbard [1927, 1930]. As the concentration of inorganic sulphate in the blood is very low, relatively large quantities of material are required for satisfactory gravimetric and volumetric analyses. It was therefore decided to investigate first the colorimetric method of Hubbard [1927, 1930]. In this method the blood-serum or blood-plasma is deproteinised with trichloroacetic acid, and the inorganic sulphate in the filtrate is then precipitated as benzidine sulphate in an acetone medium. The precipitated benzidine sulphate after solution in dilute hydrochloric acid is determined colorimetrically by the addition of hydrogen peroxide and ferric chloride, a yellow colour being produced. In their experiments with this method the present writers found that in the case of normal sera and plasma the concentration of inorganic sulphate was too low to produce a colour sufficiently intense to be of value for accurate comparison. There was, therefore, a need for a more sensitive colour reaction for benzidine.

Finally it was decided to modify the series of reactions used by Kahn and Leiboff [1928] for the determination of inorganic sulphates in urine, *viz.* diazotisation of the precipitated benzidine followed by coupling with phenol in alkaline solution. To intensify the colour the present writers have used thymol in place of phenol. The resultant colour is red.

Technique.

A solution containing 2·0071 g. benzidine hydrochloride in 500 cc. *N* HCl was prepared. Dilutions were then prepared of which 1 cc. was equivalent to 0·0025, 0·005, 0·01, 0·02, 0·04 and 0·08 mg. sulphur respectively. These dilutions were made with *N* HCl. To 2 cc. of each of these solutions 1 cc. of freshly prepared 0·1 % sodium nitrite was added. After shaking and allowing to stand 1 minute, 5 cc. of 15 % sodium hydroxide were added, and, after

thorough mixing, 5 cc. of 1 % thymol in 10 % sodium hydroxide were added to each. A red colour developed, very intense for the concentrations of benzidine chloride used. It was considered that this colour reaction would be much more suitable than the reaction used by Hubbard [1927, 1930].

Wakefield's [1929, 1] lowest standard was a dilution equivalent to 0·005 mg. sulphur, but it was found that a standard half this strength was necessary for the determination of the inorganic sulphate content of normal blood and serum. The colour obtained by coupling thymol with diazotised benzidine reached a maximum in 15 minutes and did not fade for 1 hour. The colour production was practically proportional to the amount of benzidine over this range, but in all estimations standards of varying benzidine content were prepared. In determining the inorganic sulphate content of blood, serum and plasma, all glass apparatus must be scrupulously clean and where potassium oxalate is used to prevent clotting, it must be shown to be free of all traces of sulphate.

The following technique was used for the determination of the inorganic sulphate content of blood, serum and plasma.

Reagents.

1. Acetone.
2. 20 % trichloroacetic acid in distilled water. Commercial trichloroacetic acid is purified by distillation with benzidine.
3. 0·5 % benzidine in acetone. This should be prepared fresh each day.
4. Benzidine hydrochloride standard solution.

2·0071 g. of benzidine hydrochloride are dissolved in 500 cc. *N* HCl. 1 cc. of this solution is equivalent to 0·5 mg. sulphur and is used as a stock solution. More dilute solutions for use as standards for the determination of inorganic sulphate in blood, being equivalent per cc. to 0·0025 mg., 0·005 mg., 0·01 mg., 0·02 mg., 0·04 mg. and 0·08 mg. sulphur, are prepared by diluting the stock solution with *N* HCl.

5. 0·1 % sodium nitrite solution—prepared fresh each day.
6. 15 % sodium hydroxide solution.
7. 1 % thymol in 10 % sodium hydroxide solution.

Method.

To 2 cc. of blood, serum or plasma are added 6 cc. of water and 2 cc. of 20 % trichloroacetic acid solution, and the whole is well shaken. This mixture is then either centrifuged or filtered. Most brands of filter-paper contain traces of sulphate which vitiate the results. This sulphate is insoluble in water but soluble in dilute acid solution, so that before use all filter-papers should be washed first with dilute acid, then with distilled water and finally dried in an oven. Into a 15 cc. centrifuge tube 2·5 cc. of the filtrate are measured, followed by 5 cc. of 0·5 % benzidine in acetone, and well mixed. After standing half an hour the benzidine sulphate is centrifuged and the supernatant fluid removed. The precipitate of benzidine sulphate in the centrifuge tube is washed

twice with acetone followed by centrifuging. Finally the centrifuge tube is inverted over a sheet of filter-paper until it is dry. The benzidine sulphate in the centrifuge tube is dissolved in 1 cc. of *N* HCl, with heat if necessary. After cooling, 0·5 cc. of 0·1 % sodium nitrite is added followed one minute later by 2·5 cc. of 15 % sodium hydroxide. After thorough mixing, 2·5 cc. of the alkaline thymol solution are added and after standing 15 minutes the coloured solution is compared with a suitable standard prepared at the same time. The standard is prepared as follows. 2 cc. of one of the dilute standard solutions are measured into a test-tube followed by 1 cc. of nitrite solution. After standing 1 minute, 5 cc. of 15 % sodium hydroxide solution are added, the whole is well shaken, and then mixed with 5 cc. of alkaline thymol solution. For normal blood it is only necessary to put up the lowest standard (1 cc. ≡ 0·0025 mg. S), but as a rule, to obtain standards approximating to the unknown, it is necessary to put up the whole series of standards.

As a preliminary, known amounts of ammonium sulphate were analysed in order to determine if the sulphate could be measured quantitatively by this method. The concentrations used were in the region of those sometimes found in plasma and serum, although higher than normal. These determinations were carried out under exactly the same conditions as in carrying out a determination with plasma and serum. From the results which are shown in Table I it will be seen that sulphate content could be quantitatively determined by this method with an error of less than 3 %.

Table I. *Determination of sulphate in standard solutions of ammonium sulphate.*

	mg. S per 100 cc. taken	mg. S per 100 cc. determined
1	0·91	0·90
2	1·83	1·79
3	0·91	0·93

Table II shows that added ammonium sulphate could be determined within an error of 5 %.

Table II.

	Inorganic sulphate content of blood-serum	Sulphate added	Total sulphate found	Sulphate recovered
1	1·55	1·14	2·67	1·12
2	1·55	2·28	3·95	2·40
3	1·55	4·56	6·22	4·67

The possible interference of phosphates and chlorides and of potassium oxalate, when plasma is used, must be taken into consideration. It was found, however, that 20 mg. P, 1 g. NaCl and 1 g. $(COOK)_2$ respectively per 100 cc. of blood, serum and plasma produced no appreciable effect on the determinations of sulphate.

In this work it was considered most convenient to express the sulphate content of blood in terms of S, to preserve the similarity with phosphorus and nitrogen.

The majority of workers have used serum or plasma in preference to whole blood for the determination of inorganic sulphate. Reed and Denis [1927], using a nephelometric method, state that in human blood the inorganic sulphate is found exclusively in the plasma. To test this point three different batches of normal oxalated blood were centrifuged and the cells washed twice with sulphate-free 0·85 % NaCl. 2 cc. of the corpuscular mass were mixed with 6 cc. of distilled water and then 2 cc. of 20 % trichloroacetic acid solution were added. After filtration the sulphate in the filtrate was estimated. No measurable coloration resulted. In order to make sure that the inorganic S had not dialysed through the red cell membrane the inorganic S contents of the whole blood and also of the plasma of nine different bloods were determined. Unfortunately haematocrit readings were not made but, averaging the values and assuming a cellular volume of 40 % of the whole blood, the inorganic content of the cells appears to be about one-fifth that of an equivalent volume of plasma.

Denis [1921] and Loeb and Benedict [1927] state that the retention of inorganic sulphate roughly parallels the retention of non-protein nitrogen, but Wakefield [1929, 2] states that there is no relation. It was decided to investigate this question. As a preliminary, the inorganic sulphate content of the sera of a number of normal healthy human subjects was determined. The results are shown in Table III. According to Wakefield [1929, 2] hospital patients suffering from diseases other than cardiac or renal are generally unsuitable for determining the normal range, since in many cases an increase in inorganic sulphate is observed even when there are no signs of renal inefficiency. It is extremely probable that in view of the extensive catabolic phenomena [Cuthbertson 1930, 1931], which result from injury, certain changes will occur in the blood, so that a great many hospital patients must therefore be excluded from any normal survey.

With four exceptions all the cases herein described were healthy men and women between the ages of 20 and 40 years.

Table III. *Sulphur content of normal plasma.*

	Case	Mg. S per 100 cc.	Case	Mg. S per 100 cc.	
Aet. 6 years	1	0·4	11	0·3	
13 „	2	0·4	12	0·1	
19 „	3	0·3	13	0·2	
	4	0·1	14	0·45	
	5	0·2	15	0·3	
	6	0·4	16	0·5	
	7	0·4	17	0·4	
	8	0·2	18	0·5	
	9	0·1	19	0·2	
	10	0·3	20	0·5	
			21	0·45	aet. 56

The inorganic sulphur content of the plasma or serum of normal fasting men and women appears to vary from 0·1 to 0·5 mg. per 100 cc. Other workers have obtained the following normal values expressed as mg. S per 100 cc.:

Denis [1921] plasma, 0·2–0·4 mg./100 cc.; Loeb and Benedict [1927] serum, 0·7–1·6 mg./100 cc.; Wakefield [1929] serum, 0·2–0·9 mg./100 cc.

The higher normal values quoted by Loeb and Benedict [1927] may perhaps be ascribed to the error of gravimetric estimation of amounts of barium sulphate of the order of 0·2 to 0·7 mg.

A few analyses of hospital cases are given in Table IV. They do not include any cases of obvious renal disease among their number.

Table IV.

Case	Disease	Mg. S per 100 cc.
22	Osteomalacia	0·5
23	Rheumatoid arthritis	0·2
24	Osteomalacia	0·9
25	Osteitis deformans	0·9
26	Fractured skull	0·7
27	Diabetes mellitus	0·9
28	Diabetes mellitus	0·7
29	Normal pregnancy	0·3

Forty cases of nephritis and cardio-renal disease have been investigated to determine what relation, if any, exists between the urea and inorganic sulphate content of the blood.

The urea content of the blood was determined on the whole fluid, but since this compound is contained in equal amount in plasma and red cells, the resultant values can be legitimately compared with the inorganic sulphate content of the plasma or serum.

Without considering particularly the various forms of nephritis and cardio-renal disease, but arranging the various cases in order of increasing retention of urea-nitrogen, it will be found that in general there is a coincident increase in the sulphur content and that on dividing the 40 cases into four arbitrary groups of 10 and determining the S : N ratios the values are fairly constant, 1 : 14·2, 1 : 14·7, 1 : 14·1 and 1 : 19·1. In the last group the sulphur increment lags behind the increment in urea-nitrogen (see Table V and Fig. 1).

Urea has been chosen for comparison with the inorganic sulphate, since its metabolism is in general parallel. It must be emphasised, however, that these are only average values and that the individual fluctuations are numerous and considerable. It is interesting to consider, however, that these ratios are of practically the same order as the S : N ratio of muscle, while those of the blood of normal cases are much lower (*ca.* 1 : 50).

If, however, there be deducted from these total retention values, the presumed normal content of urea-nitrogen and inorganic sulphate (15 mg. urea-N; 0·3 mg. inorganic S; S : N ratio 1 : 50), values are obtained which indicate that relative to urea-nitrogen, inorganic sulphate is retained in greater amount in the lower grades of retention, and that as the retention values increase, nitrogen relative to sulphur is retained in increasingly greater amount (S : N ratios 1 : 2·2, 1 : 8·1, 1 : 10·7 and 1 : 17·4).

From these data it appears that the measurement of the inorganic sulphate

content of the plasma or serum may prove to be of more service in the detection of mild degrees of renal impairment than the determination of the urea or non-protein nitrogen level.

Table V. *N mg. per 100 cc. whole blood; inorg. S mg. per 100 cc. plasma.*

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
30	32	13	0·3	40	58	22	1·8
31	30	15	0·6	41	52	23	2·6
32	35	15	0·8	42	40	23	0·6
33	56	15	2·3	43	63	23	1·2
34	41	16	1·6	44	63	27	1·6
35	33	17	1·0	45	70	28	0·9
36	43	18	1·8	46	80	29	4·0
37	39	20	0·4	47	60	32	1·4
38	41	20	0·9	48	59	33	2·9
39	52	22	2·3	49	80	36	2·3
Average		17	1·20			28	1·9
N : S		14·2 : 1				14·7 : 1	

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
50	56	37	2·9	60	109	60	5·1
51	87	39	3·1	61	150	70	6·0
52	69	40	3·4	62	115	83	6·1
53	64	42	2·4	63	106	88	4·2
54	79	48	4·8	64	137	100	4·2
55	71	50	2·9	65	132	111	4·9
56	82	50	2·4	66	174	120	5·7
57	112	50	2·4	67	228	132	6·3
58	100	50	2·4	68	209	158	8·9
59	109	55	5·2	69	236	177	6·1
Average		46	3·2			110	5·75
N : S		14·1 : 1				19·1 : 1	

Correlation coefficient between urea-N and inorg. S

$M_{\text{urea-N}} = 50{\cdot}25 \pm 4{\cdot}309$; $M_{\text{inorg. S}} = 3{\cdot}01 \pm 0{\cdot}212$

$\sigma_{\text{urea-N}} = 40{\cdot}405 \pm 3{\cdot}047$; $\sigma_{\text{inorg. S}} = 1{\cdot}993 \pm 0{\cdot}1503$

$r_{\text{urea-N, inorg. S}} = +0{\cdot}85 \pm 0{\cdot}028$

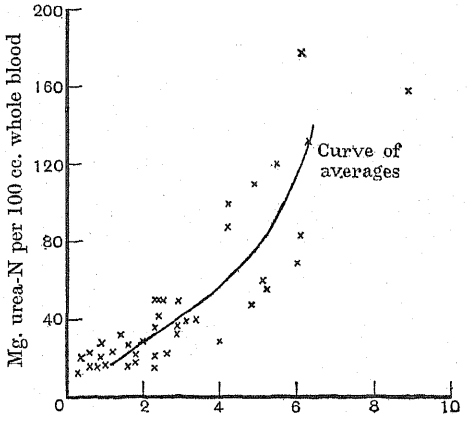


Fig. 1.

SUMMARY.

1. A method has been described for the determination of the inorganic sulphur content of the whole blood, plasma or serum. The method requires 2 cc. fluid.

2. Concentrations of phosphate, chloride and oxalate in the blood of the respective orders—20 mg. P, 1000 mg. NaCl and 1 g. $K_2C_2O_4$ per 100 cc.—do not affect the reaction.

3. There is very little inorganic sulphur in the cellular constituents of the blood.

4. The normal content for healthy adult men and women varies from 0·1 to 0·5 mg. inorganic S per 100 cc. plasma.

5. In cases of nephritis and cardio-renal disease there is on the average an increased retention of inorganic sulphur coincident with the increased retention of urea- and non-protein-nitrogen. Relative to nitrogen, inorganic sulphur is retained in proportionately greater amount in the minor degrees of retention than in the more severe types where relative to nitrogen it is less prominent.

The coefficient of correlation as will be seen from the calculation at the foot of Table V is $r_{\text{urea-N, inorg. S}} = + 0{\cdot}85 \pm 0{\cdot}028$.

In conclusion we wish to express out thanks to Dr G. M. Wishart for help with the statistical survey.

REFERENCES.

Cuthbertson (1930). *Biochem. J.* **24**, 1244.
—— (1931). *Biochem. J.* **25**, 236.
Denis (1921). *J. Biol. Chem.* **49**, 311.
Hubbard (1927). *J. Biol. Chem.* **74**, v.
—— (1930). *J. Biol. Chem.* **88**, 663.
Kahn and Leiboff (1928). *J. Biol. Chem.* **80**, 623.
Loeb and Benedict (1927). *J. Clin. Invest.* **4**, 33.
Reed and Denis (1927). *J. Biol. Chem.* **73**, 623.
Wakefield (1929, 1). *J. Biol. Chem.* **81**, 712.
—— (1929, 2). *Arch. Int. Med.* **44**, 244.
White (1923). *Amer. J. Physiol.* **65**, 537.
Yoshimatsu (1926). *Tohoku J. Exp. Med.* **7**, 553.

CXXXIX. THE SENSITISATION OF RED BLOOD CORPUSCLES IN HAEMOLYSIS.

By KSHITISH CHANDRA SEN.

From the Biochemical Laboratory, Cambridge.

(*Received July 2nd, 1931.*)

The sensitisation of red blood cells by means of a specific amboceptor and their lysis by serum complement have been known to exist for a long time, but in recent years some cases have been found where this sensitisation can be produced by chemicals, though it cannot be definitely stated whether the analogy is only superficial or not. Sachs [1908] noticed a long time ago that when washed red blood cells were treated with low concentrations of soaps and then with normal serum, a marked acceleration of haemolysis took place. No attention was apparently paid to this type of work till Ponder [1922, 1923, 1924] described certain experiments on the accelerating effect on haemolysis of normal serum and other proteins when added to a suspension of washed cells previously treated with a bile salt. This observation enabled him to give a logical explanation of the percentage haemolysis curve for taurocholate haemolysis, where a great acceleration of haemolysis takes place after the haemolytic reaction has proceeded for some time—a fact due to the effect of liberated haemoglobin. Ponder also found that histamine could replace normal serum to a certain extent in this acceleration process. Recently he [1928] has repeated the work of Browning and Mackie [1914] and of Mackie [1919] on haemolysis by brilliant green and its acceleration by serum. In several previous papers [Sen *et al.* 1929, 1931] the present author studied this acceleration of haemolysis by different substances, and the view was expressed that the acceleration of haemolysis might be due to a sudden variation of the p_H at the cell surface which then induced further physico-chemical changes. The object of this paper is to give more evidence on this point. The present position of the problem, of great importance for the theory of haemolytic reactions in general, may be stated as follows. Since red blood cells can be sensitised by treatment with bile salts, soaps or dyestuffs like brilliant green, and then show a markedly lower resistance to normal serum, the question arises as to whether the action of normal serum is a continuation of the process started by the sensitising agent or not. It is known that all these sensitising agents are more or less adsorbed by the cells and are in themselves haemolytic at higher concentrations. It is be to assumed therefore that the adsorption of these substances, though not sufficient to cause lysis within the time given on account of the low concentration used, does actually change the cell surface

in such a way that some of the components of normal serum can act haemolytically. In studying the effect of normal serum on sensitised corpuscles, we can consider separately the effect of different major constituents of the serum—namely the proteins and the salts. From the work of Sachs, Ponder and others, it appears that the protein fraction is effective. Evidence is given in this paper that under many conditions the saline fraction of the serum solutions at the same p_H level is equally effective as the normal serum itself, and often the acceleration is determined by the amount of OH^- ions added.

Experimental.

In all the experiments recorded below, sheep's erythrocytes have been used after being defibrinated and washed four times with normal saline. The concentration is expressed in terms of cc. of corpuscles per 100 cc. of saline suspension. The sensitising substance (bile salt) was also freshly prepared in saline. The time of complete haemolysis was obtained by a colorimetric comparison with a standard.

As is well known, a haemolytic experiment in presence of normal serum can be made in one of the following three ways: (1) the serum may be added to the mixture of corpuscles and bile salt (sensitiser); (2) bile salt may be added to the mixture of corpuscles and serum; and (3) a mixture of bile salt and serum may be added to the corpuscles. The effect of serum in (2) always gives an inhibition of haemolysis, in (3) there may be inhibition or acceleration, but usually inhibition, and in (1) there also may be either inhibition or acceleration depending upon several variables. All these effects due to normal serum have been imitated by using a bicarbonate-Ringer solution instead of serum. As however we are more concerned with the procedure in (1), which is analogous to the amboceptor-complement reaction, we shall only deal with this. An example of the effect of serum obtained according to (1) and (3) may be given here in order to serve as a model for the presentation of results with substances other than serum. In Table I the concentration of cells is 1 % and of taurocholate 0·3 %. In each experiment 0·5 cc. cells and 0·5 cc. taurocholate in a total volume of 5 cc. have been used.

Table I.

(1)	Time of haemolysis of the control	3 mins. 21 secs.
(2)	0·5 cc. serum + taurocholate added to cells	No haemolysis in 2 hours
(3)	0·5 cc. serum added to cells + taurocholate ½ min. after adding the taurocholate to the cells	1 min. 4 secs.

This accelerating effect was analysed further by varying the amount of serum added, determining the effect of the time interval during which sensitisation is allowed to take place, the concentration of the corpuscles and of the sensitising agent, *etc.*, and it was found that all these factors had some effect on the observed acceleration. Under certain conditions, using the same technique, an inhibition instead of acceleration could be observed. Thus Fig. 1,

in which the experiments given in Table I (3) have been repeated with varying amounts of serum added to a sensitised corpuscle mixture after ½ minute,

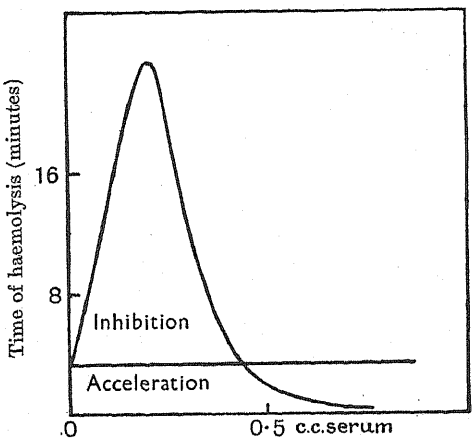


Fig. 1. Effect of serum on sensitised cells.

shows a well-marked inhibition and acceleration of haemolysis depending upon the amount of serum added. It was also found that the amount of serum necessary to induce the acceleration of haemolysis was much less when the time-interval given for sensitisation was greater. Both these effects of serum have been imitated by various substances with one common property, namely that of generating OH^- ions in solution. The experimental work falls mainly into three groups: (*a*) determination of inhibition-acceleration curves in presence of substances which give OH^- ions in solution, such as sodium hydroxide, ammonia, different amines and alkaline buffer systems; (*b*) measurements of the p_H of solutions after the addition of different amounts of these substances to sensitised cells in order to find out if there is any correlation between the change in the p_H and the observed inhibition and acceleration of haemolysis; and (*c*) the effect of time of sensitisation on the action produced by these different alkaline substances.

The general results are summarised as follows.

(*a*) If the concentration of the corpuscles and the amount of the sensitising agent (taurocholate in this case) are rightly chosen, and a suitable interval is given after which OH^- ions are added, it is easy to obtain both inhibition and acceleration with all the substances mentioned above. The concentrations can be easily adjusted by several trials. Some typical curves are shown in Figs. 2 and 3. In Fig. 2, the final cell concentration is 0·2 %, taurocholate approximately 0·01 %, and the time-interval after which ammonia is added is ½ minute. In Fig. 3 and curve *B* of Fig. 6, the cell concentration is 0·1 %, taurocholate 0·03 % and the time-interval after which dipotassium hydrogen phosphate or Ringer-bicarbonate has been added is ½ minute. It will be seen that the nature of the results is exactly the same as observed in the case of serum.

(*b*) Having determined the type of curves obtained by varying the amounts of the alkaline substances, it was decided to find out the relation, if any,

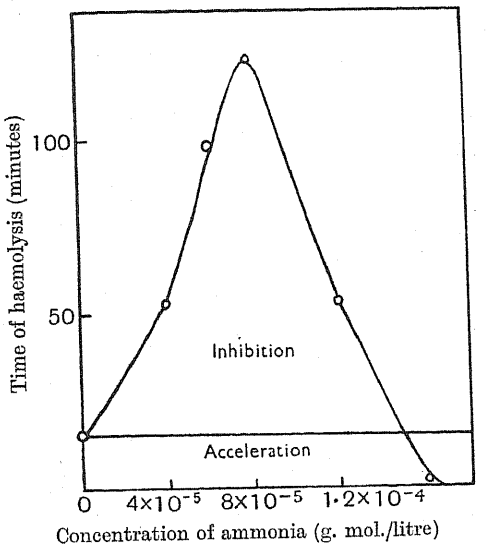


Fig. 2. Effect of ammonia on sensitised cells.

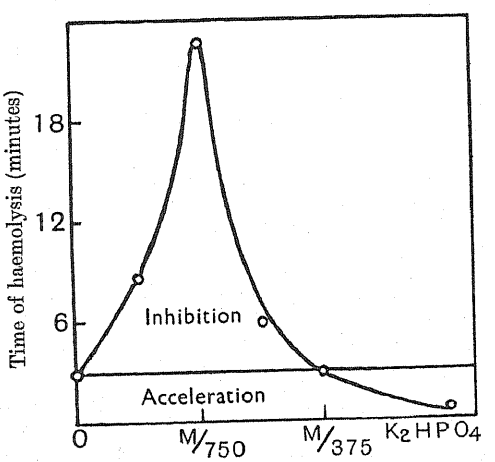


Fig. 3. Effect of dipotassium hydrogen phosphate on sensitised cells.

between the p_H change and the inhibition and acceleration of haemolysis. For this reason, the p_H of the final solutions after addition of normal serum or any of the above-mentioned substances was measured with a quinhydrone electrode. In these measurements, the final concentration of the cells was 0·1 %, and it was found that, with the concentration of haemoglobin derived from the cells, the error due to a drift in the potential could be neglected. The following solutions were used. Normal serum p_H 8·0, dipotassium hydrogen phosphate

$M/15$, Ringer-bicarbonate p_H 8·5, and 1 % gelatin in saline made alkaline with NaOH, p_H 8·5. Small amounts of these solutions were used in the actual experiments in order to vary the p_H, which was finally determined electrometrically. The corpuscle suspension had an initial p_H of 6·9 and the sensitised suspension had also almost the same p_H. In Figs. 4 and 5, two typical results

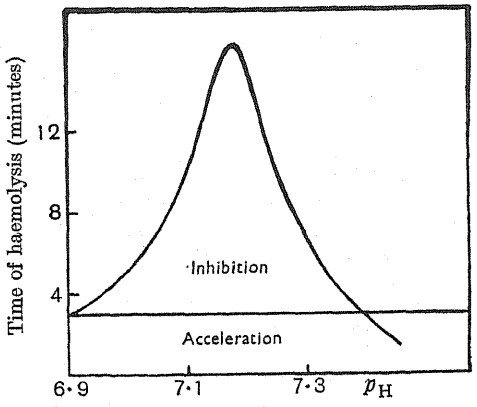


Fig. 4. Effect of p_H change on sensitised cells. Serum as accelerator.

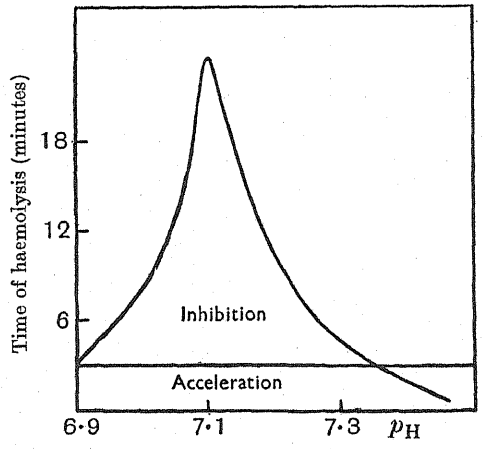


Fig. 5. Effect of p_H change on sensitised cells. Potassium phosphate as accelerator.

with serum and phosphate are shown. The curves show clearly the range of p_H over which an inhibition of haemolysis is observed. This fact, that acceleration of haemolysis only results when the p_H change is sufficiently high, seems to indicate that the lytic activity of normal serum on sensitised corpuscles is due to its alkali content. Further experiments however show that the limit of p_H beyond which the acceleration is possible is not a constant one, and the phenomenon is more complex than was thought at first.

(*c*) It has already been stated that the time-interval after which the serum or the alkaline substances are added has a definite effect on the inhibition or

acceleration of haemolysis. A typical result obtained with Ringer-bicarbonate is shown in Fig. 6. The time of complete haemolysis has been determined after

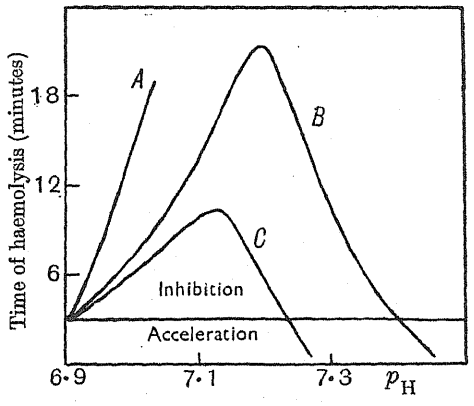


Fig. 6. Ringer-bicarbonate as accelerator. *A*. Sensitisation time, 15 secs. *B*. Sensitisation time, 30 secs. *C*. Sensitisation time, 1 minute.

allowing variable periods to elapse before the bicarbonate solution was added to cells in the course of sensitisation by taurocholate. The p_H of the solutions was measured after the completion of the experiment. The following points in this figure are to be noted. When the time of sensitisation is 15 seconds (curve *A*), the addition of bicarbonate sufficient to raise the p_H to 7·6 does not accelerate the haemolysis, but retards it. When the time of sensitisation is 30 seconds (curve *B*), an acceleration is obtained as soon as the p_H exceeds 7·4. With a time-interval of 1 minute (curve *C*), the p_H limit comes down to 7·25, and with a time interval of 2 minutes (not shown in the figure) there is practically no inhibition, and the p_H range comes down to 7·05–7·1. It is to be noted here that this last time-interval is very close to the time necessary (3 minutes) for the haemolysis of the control. I have not been able to get any acceleration below p_H 7·0 under these experimental conditions, but it cannot be stated from these results that the acceleration is determined purely by the p_H change of the medium, as the magnitude of the change is sometimes very small. It is very likely that a large change in p_H is one of the factors by which we can obtain an acceleration of haemolysis, and it seems that during the sensitisation process the surface of the cells undergoes some irreversible changes making its stability conditions highly susceptible to p_H changes.

Attention may now be drawn to the effect of H^+ ions on sensitised cells. Thus it has been found that under certain conditions, with bile salt as sensitiser, a change of p_H from 6·9 to 6·4 made by the addition of acetate buffer would give an acceleration of haemolysis. No inhibition of haemolysis by acids could however be observed. It appears therefore that in bile salt sensitisation, the surface or some particular part of the surface of the corpuscle becomes so much changed, that a variation of p_H in either direction would cause a lysis

of the cells. This effect of acid is very marked when cells are sensitised by saponin, but with normal serum no sensitisation by saponin can be observed.

In conclusion two points of interest in connection with the acceleration of haemolysis in presence of normal serum may be mentioned. It has already been stated that though a mixture of normal serum and bile salt usually shows an inhibition of haemolysis, yet under some conditions this mixture might have a greatly increased haemolytic power. This fact of course is not solely dependent on the proteins, as a similar effect has been observed with a mixture of bile salt and either ammonia or ethylamine. The conditions under which this greatly increased haemolytic efficiency is observed have not been studied thoroughly, but it appears that there are at least three variables in this, namely, concentration of the cells, concentration of the bile salt and the concentration of serum or the alkaline substance used as the inhibitors. The second point of interest is the the fact that though serum and certain other substances will haemolyse a sensitised suspension and some variables in this reaction have been mentioned, there is another variable, namely the condition of the cells themselves, which profoundly affects the results. This point has been previously emphasised by Ponder.

Summary.

1. In a study of the effect of normal serum on corpuscles sensitised by bile salts, it is found that both inhibition and acceleration of haemolysis can be observed under suitable conditions.

2. It is found that this effect of normal serum is not solely due to the proteins, but the alkali-content of the serum must have some effect.

3. This effect of serum has been imitated by many substances which yield hydroxyl ions in solution.

4. Erythrocytes can be highly sensitised towards acids by previous treatment with bile salt or saponin.

5. The acceleration of haemolysis of corpuscles sensitised by bile salts due to the addition of serum or other acid or alkaline substances cannot be considered to be entirely dependent on the p_H change of the solution. The time allowed for the sensitisation to take place has a great influence on the effect of p_H change and acceleration of haemolysis.

6. Normal serum acts both as a haemolytic agent and an anti-haemolyte depending on the conditions of the experiment, and it appears that the haemolysis of sensitised cells by serum will have to be considered as a continuation of the action started by the sensitising substance.

I am much indebted to Sir F. G. Hopkins for his kind interest in this work.

REFERENCES.

Browning and Mackie (1914). *Z. Immunität.* **21**, 422.
Mackie (1919). *Trans. Roy. Soc. South Africa*, **8**, 59.
Ponder (1922). *Proc. Roy. Soc. Lond.* B **93**, 86.
—— (1923). *Proc. Roy. Soc. Lond.* B **95**, 382.
—— (1924). Erythrocytes and the action of simple haemolysins. (Edinburgh.)
—— (1928). *Proc. Roy. Soc. Lond.* B **103**, 556.
Sachs (1908). *Biochem. Z.* **12**, 278.
Sen and Mitra (1929). *J. Ind. Chem. Soc.* **6**, 155.
—— and Roy (1931). *Ind. J. Med. Research*, **18**, 881.
—— Roy and Mitra (1929, 1). *Nature*, **124**, 304.
—— —— —— (1929, 2). *J. Ind. Chem. Soc.* **6**, 489.

CXL. THE OXIDATION OF OLEIC ACID BY MEANS OF HYDROGEN PEROXIDE WITH AND WITHOUT THE ADDITION OF COPPER SULPHATE: A POSSIBLE ANALOGY WITH ITS OXIDATION *IN VIVO*.

By IDA SMEDLEY-MACLEAN
and MARGARET SARAH BEAVAN PEARCE.

From the Biochemical Department, Lister Institute, London.

(*Received July 3rd, 1931.*)

The discussion of the method of biological oxidation of the fatty acids has hitherto been almost entirely confined to the saturated acids, in spite of the fact that the acid which occurs in greater proportion than any other in the large majority of fats is the unsaturated oleic acid. Such facts as are available point to the conclusion that where oxidation is taking place rapidly the fat contains a relatively large proportion of unsaturated acid. Lewkowitsch quotes figures, for instance, showing that the fat of animals in the wild state is more unsaturated than that of the same species reared in domesticity: the iodine value of the fat of the wild rabbit is 101 as compared with 64 for the fat of a tame rabbit, and similar differences hold for the goose and the hog. Although it has been established [Ellis and Zeller, 1930] that carbohydrate feeding leads to a diminution of the unsaturated acids in the storage fat, it is uncertain whether variations in diet would account for the whole of the above differences, and it is possible that where the animal is leading a more active life a higher proportion of unsaturated acids is available. In the fat of the lower organisms the proportion of unsaturated acid is greater when they are grown at a low temperature and diminishes as the temperature at which they are grown is raised [Terroine, *et al.* 1927; Pearson and Raper, 1927]. Blood-plasma contains a high proportion of unsaturated acids, a large proportion of which are more unsaturated than oleic acid [Bloor, 1924]. In the liver oils the proportion of saturated acids is comparatively low. Bloor [1926, 1927] has shown that in the fat extracted from heart muscle and from voluntary muscle it is the unsaturated acids which predominate, the proportion being greater than in storage fat. The oxidation of the unsaturated acids in the body appears to be a normal process and it is generally held, largely owing to the work of Leathes and his colleagues, that desaturation of the fatty acids precedes oxidation. Nothing, however, is known of the stages by which oleic acid breaks down in the body and the theory of β-oxidation, which has found most acceptance as the probable method by which the saturated acids containing normal chains

are oxidised, does not necessarily hold good in the case of the unsaturated acids, since here oxidation must first take place at the ethylenic linkage.

In the laboratory the oxidation of oleic acid by the action of potassium permanganate has been carefully investigated. Saytzeff [1886] showed that dihydroxystearic acid was produced when oleic acid was treated with a dilute alkaline solution of permanganate. Lapworth and Mottram [1925], working with the same reagent, found that, if the permanganate solution were sufficiently dilute and the temperature between 0° and 10°, the conversion of oleic acid to 9 : 10-dihydroxystearic acid was almost quantitative.

By the further action of permanganate Edmed [1898] obtained nonoic, azelaic and oxalic acids, and previously Spiridonoff [1887] using the same reagent had isolated sebacic, suberic and caprylic acids. Lapworth and Mottram [1925] reconciled these results and showed that the oxidation of the dihydroxystearic acid first formed can take place in two ways according to the following equations:

(1) $CH_3.(CH_2)_6.CH_2.CH(OH).CH(OH).CH_2.(CH_2)_6.COOH + 3O =$
$CH_3.(CH_2)_6.CH_2.COOH + COOH.CH_2.(CH_2)_6.COOH + H_2O.$
pelargonic acid azelaic acid

(2) $CH_3(CH_2)_6.CH_2.CH(OH).CH(OH).CH_2.(CH_2)_6.COOH + 8O =$
$CH_3.(CH_2)_6.COOH + COOH.COOH + COOH.(CH_2)_6.COOH + 2H_2O.$
caprylic acid oxalic acid suberic acid

The latter reaction is favoured by a more dilute solution and a lower temperature.

It is certainly improbable that an unsaturated acid containing an ethylenic linkage in the 9.10-position would in the first instance undergo oxidation in the β-position. Such migrations of the double bond as are known to take place in the fatty acids are only brought about by violent chemical reagents often acting at high temperatures. A shift from the 9.10- to the 3.4-position, for instance, is brought about when oleic acid is treated with concentrated sulphuric acid, giving rise to stearolactone, and when oleic acid is fused with caustic potash [Edmed, 1898; Le Sueur, 1901] a change of the unsaturated linkage from the 9.10- to the 2.3-position must be involved, since palmitic and acetic acids are obtained. Although this conversion of oleic to palmitic acid is exceedingly interesting, it is difficult to regard a process brought about by fusion with caustic potash as affording grounds for believing that such a change is brought about by enzyme action at the body temperature. When laboratory experiments are used as a basis for suggesting analogies with the processes actually occurring in the body, it is usual to choose reactions which can be carried out at a comparatively low temperature by the aid of mild chemical reagents such as might conceivably be present in the body. Such a method is the oxidation with hydrogen peroxide studied by Dakin and other workers, although in the case of the higher fatty acids Dakin found it necessary to carry out the reaction at 95°. Even at this temperature only about 10 % of the original acid is attacked, but Dakin [1908, 1910] succeeded in isolating the

ketones derived from the β-hydroxy-derivatives of the fatty acids by the elimination of CO_2. He quoted these experiments as furnishing an instance of oxidation under conditions not too far removed from those obtaining in the body and advanced them in support of the view that the degradation of the saturated fatty acids takes place in the body by a repeated process of β-oxidation. However, if the β-keto-acids are formed in the body, all except β-ketobutyric acid must be immediately further broken down without the separation of the corresponding ketones, since the only fatty ketone known to occur in the body is acetone, and there is no evidence that the higher ketones would be readily attacked.

The possible conversion of the β-hydroxy-acids to αβ-unsaturated acids also lacks any experimental foundation.

Clutterbuck and Raper [1925] have shown that in the action of hydrogen peroxide on the saturated fatty acids, oxidation occurs in the γ- and δ-positions at the same time as in the β-position and to about an equal extent.

Hazura [1888] investigated the oxidation of oleic acid with hydrogen peroxide in the absence of an alkali at ordinary temperature but failed to detect any evidence of oxidation.

The present communication records the results of the oxidation of this acid under the experimental conditions used by Dakin and his colleagues and also by the method described by Battie and Smedley-MacLean [1929] in which, by the addition of a cupric salt as catalyst, oxidation proceeds much more readily and at a lower temperature.

The oxidation of oleic acid with hydrogen peroxide at 95°.

4 g. of oleic acid were used in each experiment, the oxidation being carried out in slightly alkaline solution and a sufficient quantity of the peroxide added to furnish four atoms of oxygen for each molecular proportion of oleic acid. The reaction mixture was heated at 95° for 3 to 3·5 hours. Acid was then added and the CO_2 blown off and absorbed by baryta, and the separation into various fractions carried out as described on p. 1260. After the carbonic acid had been removed, the insoluble oil floating on the surface was filtered off: this formed nearly 90 % by weight of the oleic acid originally taken.

The carbonic, acetic and formic acids produced account for less than 10 % of the carbon of the oleic acid originally present. The neutral volatile distillate gave a positive reaction with sodium nitroprusside, which was probably due to acetone since we succeeded in isolating a very small amount of crystals of a dinitrophenylhydrazone which melted at 121–123°. A specimen of acetone dinitrophenylhydrazone melted at 123–124°. A trace of acetone was, therefore, probably present. A small amount of acid (0·16 g.) was obtained from the aqueous reaction mixture by continuous extraction with ether. There was too little to be purified satisfactorily, but since it gave an iodoform reaction in the cold and a molecular weight of 151 by titration, it was evidently similar to the 4-keto-7-hydroxycaprylic acid isolated in much larger quantity in subsequent

experiments when copper had been added to the reaction mixture. We were unable to detect succinic acid in the small amount of material available but we can say with certainty that not more than a trace of this acid could have been present. A trace of an acid soluble in water and insoluble in ether appeared to be oxalic acid.

The main fraction of the oil was insoluble in water. It was neutralised with $N/2$ NaOH and the aqueous solution extracted with ether to remove any neutral substances present.

The neutral fraction was present only in small amount, the total weight being about 5 % of the oleic acid originally taken. It was separated into two parts by gently warming its alcoholic solution on a water-bath and adding to it at intervals small quantities of $N/2$ NaOH until the liquid remained just alkaline. After removing the alcohol the slightly alkaline solution was extracted with ether and a small amount of a neutral substance isolated. Four specimens of the fraction, which did not dissolve on warming with dilute alkali, were obtained in different experiments and were analysed with the following results:

	% C	% H	
(4)	75·70	11·90	
(3)	75·25	11·96	
(2)	74·71	11·65	
(1)	74·25	11·79	(least pure specimen, probably contaminated by lactone)

These figures agree best with the figures required by a diketoheptadecane $C_{17}H_{32}O_2$ (C, 76·12; H, 11·90 %). Attempts to prepare an oxime or dinitrophenylhydrazone were, however, unsuccessful and the nature of this substance has not been satisfactorily settled. The only possible product of β-oxidation would, however, be contained in this fraction, so that we can say that if under the conditions of this experiment β-oxidation does take place, it can occur only to a very slight extent and is certainly not the main path of the reaction.

Acidification of the solution produced by warming the neutral fraction with dilute alkali set free an oil with a pleasant characteristic odour, which was left in a desiccator until of constant weight and then analysed. It contained C, 69·70, 69·79; H, 10·49, 10·71 %, thus agreeing with the figures required by the lactone of a ketodihydroxystearic acid, $C_{18}H_{32}O_4$, C, 69·23; H, 10·25 %. Oxidation had, therefore, probably taken place in the γ- or δ-position as well as at the ethylenic linkage.

The acid fraction was extracted by means of light petroleum from the aqueous solution after it had been acidified; a small fraction remained insoluble in light petroleum from which crystals were obtained melting at 130°; these were identified as dihydroxystearic acid by a determination of a mixed melting-point. The composition of the oil which dissolved varied somewhat in different experiments; its iodine value varied between 48 and 58, so that if it is justifiable to regard this as due to the presence of oleic acid, from 53 to 65 % of the fraction consisted of unchanged oleic acid, *i.e.* from 40 to 50 % of the starting material had not undergone any oxidation. The presence of

oleic acid in the fraction was confirmed by oxidation with a dilute solution of potassium permanganate at 5° and the resulting dihydroxystearic acid was identified by analysis and determination of melting-point.

It was found that a separation of the fraction might be made by means of its silver salts. The oil was dissolved in alcohol, neutralised, the silver salt precipitated in boiling solution, filtered off and well washed with hot water. The insoluble silver salt was then repeatedly heated with boiling water, and the washings added to the solution of the soluble silver salt, which was then acidified and extracted with ether. The oil obtained in two separate experiments contained C, (1) 64·69, 64·65; (2) 63·01: H, (1) 9·70, 9·78; (2) 9·29 %. Mol. wt. by titration = (1) 188; (2) 198. Iodine value = 0. Calculated for ketodecoic acid, $C_{10}H_{18}O_3$, C, 64·52; H, 9·68 %. Mol. wt. = 186: for ketononoic acid C, 62·77; H, 9·42 %. Mol. wt. = 172.

A silver mirror was deposited on the sides of the tube when a solution of the sodium salt was warmed with an ammoniacal solution of silver nitrate. When heated with silver oxide and water, CO_2 was evolved and a volatile acid with a slightly goaty smell deposited on the sides of the tube. An α-ketoic acid is therefore present, a derivative of either nonoic or decoic acid. It was thought possible that the semialdehyde of azelaic acid might be present in this fraction, but no evidence was obtained of the presence of azelaic acid on oxidation.

The acid obtained from the insoluble silver salt contained from 71 to 75 % C and from 10·6 to 11·8 % H. From this fraction a very small amount of white crystals was separated melting at 49–50°, which appeared to be identical with those obtained in another series of experiments (see p. 1260), and which gave figures on analysis approximating to those required by ketostearic acid. The fraction obtained from one experiment (C, 74·03; H, 11·32 %) was converted into the zinc salt and this fractionally crystallised from boiling alcohol. The acid obtained from the least soluble portion gave figures on analysis agreeing with those for oleic acid: the composition of that from the most soluble part of the zinc salt agreed with that of a ketostearic acid (C, 72·55, 72·53; H, 10·61, 10·80 %) but, since it had an iodine value of 41, it still contained a considerable proportion of oleic acid, and we were unable to obtain from it either a satisfactory oxime or dinitrophenylhydrazone, nor did crystals separate on standing.

An attempt to calculate the composition of the oxidation product was made as follows. The acid derived from the soluble zinc salt contained 72·54 % C and 10·7 % H, and had an iodine value of 41. If the latter were due to oleic acid (C, 76·6; H, 12·05 %; I.V. 90), the following equations would give the percentage of carbon and hydrogen in the oxidation product.

$$41 \times 76{\cdot}6 + 49\ \mathrm{C} = 90 \times 72{\cdot}54, \text{ whence } \mathrm{C} = 69{\cdot}1\ \%$$
$$41 \times 12{\cdot}05 + 49\ \mathrm{H} = 90 \times 10{\cdot}7, \text{ whence } \mathrm{H} = 9{\cdot}6\ \%.$$

These figures agree most nearly with those required for the diketostearic

acid (C, 69·23; H, 10·26 %), so that although we have not yet succeeded in isolating this substance, it is possible that it is present.

Table I. *Results of oxidation of 4·05 g. oleic acid as Na salt by 33 cc. (20 vol.) H_2O_2 in absence of a catalyst.*

Wt. CO_2 g.	Volatile acids g.	Volatile neutral. Rothera's acetone test	Fraction water-ether-soluble g.	Fraction water-soluble ether-insol. g.	Fraction water-insol. ether-sol. g.	
					Acid	*Neutral*
0·10	Formic 0·09 Acetic 0·48	Positive	(*a*) 0·11 water-sol. acid (*b*) 0·05 alcohol-sol. acid	0·07	*Petroleum-soluble:* (*a*) oleic acid 1·71 (*b*) diketostearic acid ? 0·81 (*c*) α-ketodecoic or α-keto-nonoic 0·28 *Petroleum-insoluble:* Dihydroxystearic 0·46	0·3

In his work on the oxidation of the saturated fatty acids, Dakin found that the amount of oxidation was very little affected by increasing the proportion of hydrogen peroxide. In the series of experiments about to be described in which a cupric salt was used as catalyst, a large excess of peroxide was taken and an experiment was therefore carried out in which the same excess of peroxide was used but without the addition of the catalyst. Sufficient peroxide was now present to oxidise completely the whole of the oleic acid to carbonic acid.

Carbonic, formic and acetic acids now accounted for about 18 % of the carbon of the original oleic acid: the chief product was still a colourless oil, but the greater proportion of it was now insoluble in light petroleum. The proportion of dihydroxystearic acid was much greater and more of the oleic acid had undergone oxidation.

To sum up, whereas in Dakin's investigations on the higher saturated fatty acids about 10 % of the acid was attacked, the chief products being carbonic acid and the methyl alkyl ketone formed as decomposition products of the corresponding β-hydroxy-acid, when oleic acid is similarly treated about 50 % of the acid undergoes oxidation, a considerable proportion of it being converted to a fraction corresponding in composition with diketostearic acid; α-ketodecoic acid is formed and small amounts of dihydroxystearic acid. Carbonic, formic and acetic acids account for less than 10 % of the total carbon of the original oleic acid. Less than 5 % of the oxidation product consists of a neutral fraction in which a lactone of ketodihydroxystearic acid and an oil having the empirical formula required for a diketoheptadecane were present. The latter substance was not, however, definitely identified.

The oxidation of oleic acid with hydrogen peroxide in presence of a cupric salt.

When the oxidation of oleic acid is carried out in the presence of a cupric salt, the results differ widely from those just described. Oxidation proceeds

much more rapidly and at 95°, the temperature at which Dakin carried out his experiments, the carbonic acid formed accounts for the complete oxidation of more than 50 % of the total oleic acid, another 20 % being converted to acetic and formic acids. 70 % of the original acid has, therefore, been broken down to substances containing one or two carbon atoms. The fraction of residual oil which alone contains any product of a molecular weight approaching that of oleic acid is very small. In two experiments, in each of which 4 g. of oleic acid were taken, the residual oils insoluble in water were respectively 0·03 and 0·13 g. as compared with 3·7 g. in the corresponding experiment without the catalyst. Even at 60° the amounts of carbonic, formic and acetic acids correspond to a complete conversion of 20 % of oleic acid into these substances. At this temperature, the fraction derived from the aqueous reaction mixture by continuous extraction with ether contains the largest portion of the product, *i.e.* about 2·5 g. from 4 g. oleic acid, equivalent to the conversion of 40 % of the oleic acid. This fraction consists mainly of two substances:

(*a*) Crystals melting at 181–182° (0·5 g.) after being once recrystallised from water.

Analysis: C, 41·53; H, 5·23 %. Mol. wt. (by Ag salt), 132.
Calculated for $C_4H_6O_4$: C, 40·68; H, 5·08 %. Mol. wt. 118.
This substance was therefore identified as succinic acid.

(*b*) An oil (1·7 g.) to which the formula $C_8H_{14}O_4$ was assigned. Microanalysis of specimens obtained in two different experiments gave the following results:

C, 53·66, 53·41; H, 7·83, 7·97 %.
Mol. wt. (*a*) by titration, 166; (*b*) by iodoform estimation, 174, 189.

It was difficult to free this substance from all traces of succinic acid and the latter probably accounts for the low carbon percentage. The same substance was, however, subsequently obtained in a purer condition from the oxidation of caprylic acid (see p. 1259). A mixture of 90 % of the above acid with 10 % succinic acid would give 53·72 % C, 7·75 % H. Mol. wt. 162.

The formula $C_8H_{14}O_4$ was therefore adopted and the acid regarded as a 4-keto-7-hydroxycaprylic acid for the following reasons.

(1) It gave an iodoform reaction in the cold. This would be produced by the presence of a —CO— or —CH(OH)— group next to the terminal methyl group and its formation shows that oxidation must have occurred at the seventh carbon atom.

(2) The estimation of the $CH_3.CO$— group by its conversion to iodoform corresponded with a molecular weight of 174 and 189.

(3) The amount of substance extracted by ether from the reaction mixture when the experiment had been carried out at 95° was small but consisted chiefly of succinic acid. The product when the oxidation was carried out at 60° consisted mainly of the ketohydroxycaprylic acid, the proportion of succinic acid present being small. Some of the caprylic acid derivative

appeared, therefore, to have been converted into succinic acid at the higher temperature.

$CH_3.CH(OH).CH_2.CH_2.CO.CH_2.CH_2.COOH \rightarrow COOH.CH_2.CH_2.COOH.$

(4) In the oxidation of caprylic acid itself at 60° under similar conditions the same ketohydroxycaprylic acid and succinic acid were formed, so that in this case succinic acid must have come from the eight-carbon atom chain.

(5) The acid was not readily converted to a lactone and the hydroxyl group is not therefore in the γ- or δ-position.

(6) The presence of the —CO— or —CH(OH)-group in the α- or β-position could be excluded.

The amount of the ketohydroxycaprylic acid isolated when the oxidation was carried out at 60° corresponded to the conversion of almost exactly 70 % of the 8 terminal atoms of the oleic chain and it is improbable that the separation of this oxidation product was quantitative: it seemed most probable, therefore, that caprylic acid was formed from the oleic acid and subsequently underwent oxidation. The oxidation of caprylic acid was carried out under similar conditions and from the corresponding fraction of the product the same substance $C_8H_{14}O_4$ was isolated, together with succinic acid.

Its properties agreed with those described above.

Micro-analysis: C, 54·83; H, 7·57 %.

Calculated for $C_8H_{14}O_4$: C, 55·17; H, 8·05 %.

Mol. wt.: from titration 172; from an iodoform estimation 172. Theory 174.

Table II. *Showing results of the oxidation of 5 g. caprylic acid at 60° for 4 hours. Materials used; 32 cc. N NaOH, 110 cc. water, 15 cc. solution of $CuSO_4.5H_2O$ (2·73 %) and 350 cc. H_2O_2 (20 vols.).*

Product	Exp. I g.	Exp. II g.	Approximate % of total carbon calc. on mean of I–II	
Carbon dioxide:				
(*a*) during oxidation	0·50	0·33	—	
(*b*) during distillation	0·28	0·35	6·1	
Volatile acids:				
Caprylic (recovered)	0·37	0·55	9·3	Mol. wt. by titration {147 / 155
Acetic, formic	1·03	0·69	10·4	Mol. wt. by titration {79 / 49
Soluble in water and in ether:	3·99	3·25	59·8	Calc. as containing 55 % C
Succinic	1·80	—	—	Small amount crystals
Ketohydroxycaprylic				M.P. 178–180°. For analysis of ketohydroxycaprylic acid see above
Sparingly sol. in water, sol. in alcohol	1·03	—	—	Contains a lactone
Soluble in water, insoluble in ether	0·40	0·18	2·4	Mol. wt. (dibasic) 82. Glycollic and glyoxylic absent. Chiefly oxalic
Insoluble in water, soluble in ether:				
(*a*) residual oil	0·10	0·70	6·5	Mol. wt. by titration {178 / 171
(*b*) extracted by ether from charcoal	0·14	0·14	—	Titrates as lactone
		Total	94·5 of total carbon	

When the oxidation of the oleic acid was carried out at 60° about 20 % of it was converted to a mixture of oxidised derivatives of stearic acid, varying somewhat in different experiments. These were separated into products respectively insoluble and soluble in light petroleum, and the following fractions were separated and analysed. The residual oil varied in composition in different experiments and we did not obtain sufficient completely to purify the various fractions, or to identify them satisfactorily.

Table III. *Composition of fractions of water-insoluble oil obtained by oxidation of oleic acid at 60° in presence of a cupric salt, determined by micro-analyses.*

(1) Crystals M.P. 48–50°, soluble in light petroleum
C, 73·57; H, 11·73 %. Mol. wt. by titration 302.
Calculated for ketostearic acid: C, 72·50; H, 11·41 %. Mol. wt. 298.
M.P.: 9-ketoacid 83°; 10-ketoacid 74–76°

(2) Crystals M.P. 84–86°, insoluble in light petroleum
C, 67·44; H, 11·25 %.
Calculated for dihydroxystearic acid: C, 68·34; H, 11·39 %.
M.P.: low-melting form 98–99°; high-melting form 130°

(3) Oil soluble in light petroleum
C, 67·59; H, 10·41 %

(4) Oil insoluble in light petroleum
C, 66·58; H, 9·98 %. Mol. wt.: acid from Ag salt 348; by titration 358. Oil titrates like a lactone.
Calculated for lactone of ketotrihydroxystearic acid: C, 65·87; H, 9·79 %. Mol. wt. acid 346

(5) Oil
C, 61·48; H, 9·98 %

Method of experiment.

4 g. of pure oleic acid were neutralised with 95 % of the theoretical amount of *N* NaOH, and dissolved in 210 cc. of water to which 24 cc. of a 2·73 % solution of $CuSO_4$, $5H_2O$ had been added. The solution was contained in a three-litre flask fitted with a reflux condenser, to the top end of which was connected (*a*) an inlet tube provided with a clip through which air or nitrogen could be passed into the liquid in the flask, and (*b*) an exit tube leading to a series of flasks containing known volumes of standard baryta solution. This was then heated on a water-bath at the required temperature. 560 cc. of 20 volume hydrogen peroxide, previously made neutral, were added, and the flask well shaken. The inlet tube was closed and the CO_2 evolved collected in the baryta flasks. After 3 to 4 hours the heating was stopped, 5 cc. of concentrated HCl or H_2SO_4 were added to the reaction mixture and a current of nitrogen passed through to drive off CO_2, which was absorbed by the standard baryta.

The reaction mixture was filtered through a wet filter-paper which was subsequently dried and extracted with ether, any oil insoluble in water being thus separated. The filtrate was made alkaline to litmus, charcoal added and left to stand overnight so that any excess of hydrogen peroxide was destroyed. The charcoal was removed by filtration and when dry extracted with ether to remove any product which it might have absorbed. The filtrate was acidified

Table IV. *Showing results of oxidation of oleic acid by H_2O_2 in presence of a cupric salt.*

	CO_2		Volatile acids		Volatile neutral. Na nitroprusside test for acetone	Extract sol. in water and in ether g.	% of total C	Extract sol. in water, insol. in ether g.	% of total C	Oil insol. in water, sol. in ether g.	% of total C
At 60°	Wt. (g.)	% of total C	g.	% of total C							
Exp. I. 3½ hrs.											
4 g. oleic acid as Na salt, 560 cc. H_2O_2 (20 vol.)	During oxid. 0·67 ,, dist. 0·30 0·97	8·6	Formic 0·10 Acetic 0·68	9·7	Positive	Total 1·56 *Easily sol. in water* 0·92 (*a*) 4-keto-7-hydroxy-caprylic acid (*b*) succinic acid *Sol. in alcohol* 0·38	—	Total 0·49 (contains oxalic and glycollic)	4·6 approx.	Total 0·86	18·9 approx.
Exp. II. 3½ hrs.											
4 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 1·04 ,, dist. 0·37 1·41	12·6	Formic 0·09 Acetic 0·65	9·4	Positive	Total 2·46 *Easily sol. in water* 2·15 (*a*) ketohydroxycaprylic acid 1·69 (*b*) succinic acid 0·46 *Sol. in alcohol* 0·10	36·2	Total 0·76	7·2 approx.	Total 0·83	18·2
Exp. III. 3½ hrs.											
4·86 g. oleic acid as NH_4 salt, 560 cc. H_2O_2	Not estimated	—	Formic 0·25 Acetic 1·11	13·7	Positive	Total 2·79 *Easily sol. in water* 2·59	—	Total 0·83	6·5 approx.	Total 0·63	11·4
Exp. IV. 5 hrs.											
4·26 g. oleic acid as NH_4 salt, 560 cc. H_2O_2	During oxid. 0·65 ,, dist. 0·22 0·87	7·2	Formic 0·08 Acetic 0·79	9·7	Positive	Total 2·93	—	—	—	Total 0·53	10·9
At 95°											
Exp. V. 3½ hrs.											
4·1 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 5·75 ,, dist. 0·45 6·20	53·9	Incompletely acidified, not estimated	—	Positive faint	Total 0·40 *Easily sol. in water* 0·25 *Sol. in alcohol* 0·15	—	Total 0·27	2·5 approx.	Total 0·13	2·8
Exp. VI. 3½ hrs.											
4·03 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 5·03 ,, dist. 0·55 5·58	50·5	Formic 0·01 Acetic 1·45	18·6	Positive	Total 0·76 *Easily sol. in water* 0·55 (*a*) ketohydroxy-caprylic acid 0·07 (*b*) succinic acid 0·48 *Sol. in alcohol* 0·09	7·6	Total 0·33	3·1 approx.	Total 0·03	0·7

and steam-distilled, the apparatus being arranged so that any CO_2 given off could be absorbed and estimated.

The distillate was neutralised and distilled to remove any neutral volatile products, the neutral distillate being tested for acetone and aldehyde. The remaining solution was evaporated to dryness and the sodium salts of the volatile acids obtained. The percentage of formate in this was estimated by the mercury method. After steam distillation, the residual non-volatile liquor was evaporated to a small bulk and subjected to continuous ether extraction for several days. The total extract obtained was evaporated to dryness and dried in a vacuum desiccator until of constant weight. The golden-brown oil remaining was extracted first with water and then with 96 % alcohol, and the residues from these two extracts were separately examined.

The aqueous solution, after it had been thoroughly extracted with ether, was treated with H_2S to remove traces of copper and then evaporated to dryness and extracted with absolute alcohol. The residue contained in the alcohol was weighed and examined for glycollic, glyoxylic and oxalic acids.

Discussion.

When oleic acid is treated with hydrogen peroxide in the presence of a copper catalyst, it appears that oxidation first takes place at the 9th and 10th carbon atoms and then proceeds further in the 18 carbon chain, the position of the carbon atoms next attacked not having been definitely determined; since a lactone was obtained it seems probable that oxidation had taken place in the γ- or δ-position.

The oxidised chain then breaks up, but neither azelaic nor suberic acid could be detected. Succinic and oxalic acids were the only dibasic acids isolated, only a trace of the latter being present.

The eight terminal carbon atoms of the oleic chain are broken off and the remaining part of the chain appears to be completely broken up, succinic, carbonic, formic, acetic, glycollic and oxalic acids having been identified. The amount of succinic acid formed during the oxidation must be considerably greater than that actually isolated, for it has been shown that in an acid reaction mixture succinic acid is rapidly oxidised under the conditions of the experiment [Battie and Smedley-MacLean, 1929]. During the oxidation of the oleic acid in alkaline solution probably comparatively little of the succinic acid is attacked, but after the acidification of the reaction mixture, excess of the peroxide still being present, considerable oxidation of the succinic acid will occur while the carbonic acid is being removed from the solution.

The eight terminal atoms of the oleic chain undergo oxidation, behaving apparently as caprylic acid itself behaves under similar conditions, and the 4-keto-7-hydroxycaprylic acid first formed is broken down at a higher temperature, there being evidence that succinic acid is formed as a product of this decomposition.

In the absence of the copper catalyst it is possible that a very small amount

of a ketone may have been formed such as would be expected if the ketostearic acid first formed had undergone β-oxidation, but the evidence for it was not definite, and in any case the amount was very small. The direct evidence for the β-oxidation of the higher fatty acids rests exclusively on experiments on the oxidation of stearic, palmitic and myristic acids by means of hydrogen peroxide at a temperature very little below the boiling-point of water. Experiments furnishing evidence that the animal body can directly bring about β-oxidation have all been carried out with acids containing ten or less carbon atoms. The experiments of Embden and Marx [1908], in which the liver was perfused with solutions of salts of the lower fatty acids containing up to ten carbon atoms, showed that one molecular proportion of the even-numbered fatty acids produced from 10 to 17 % of a molecule of acetone. The highest proportion (17 %) was produced from a molecular proportion of butyric and decoic acids respectively, which is in accordance with the view that one molecule of acetoacetic acid comes from one molecule of fatty acid, being derived probably from the four terminal carbon atoms. Actually, small amounts of acetone were also derived from the odd-numbered acids, a molecular proportion of nonoic acid furnishing 4 % of a molecule of acetone.

Again, the classical feeding experiments of Knoop were carried out with acids containing not more than six carbon atoms.

Leathes has urged that the first stage in the utilisation of the fatty acids consists of their desaturation in the liver. The results of the experiments *in vitro* now described show that when oleic acid is oxidised under these comparatively mild conditions, an eight-carbon chain fatty acid remains intact and undergoes further oxidation, but that the chain from the carboxyl group to the double bond is completely broken down into substances containing not more than two carbon atoms with the possible exception of succinic acid. The actual isolation from oleic acid of caprylic acid derivatives disposes of the objection raised by Leathes and Raper [1925, p. 194] that oleic acid would first be broken down into pelargonic and azelaic acids, and that as these contain odd numbers of carbon atoms they would not be expected to furnish acetoacetic acid.

The evidence now presented from laboratory experiments has been obtained under conditions probably more nearly approaching those existing in the body than those used in previous work on the oxidation of oleic acid. The action of a mild oxidising agent such as hydrogen peroxide at 60° is not very far removed from conditions *in vivo* and even copper is known to occur in marked amount in liver tissue [Morrison and Nash, 1930].

The fact that the introduction of a cupric salt as catalyst makes such a marked difference in the course of the oxidation of fatty acids by hydrogen peroxide, in the case of caprylic acid replacing the β-oxidation described by Dakin by γ-oxidation and in the case of caprylic and oleic acids producing a considerable proportion of succinic acid, is of some interest when its biological application is considered.

The wide distribution in the body of the powerful specific enzyme succinoxidase [Batelli and Stern, 1911; Thunberg, 1909] has so far found no sufficient explanation, for, as Clutterbuck [1927] has pointed out, its only function appears to be to deal with the comparatively small amount of succinic acid derived from glutamic acid.

The conversion of 50 % of butyric into succinic acid [Cahen and Hurtley, 1917], the production of γ- and δ-keto-acids from the higher saturated fatty acids [Clutterbuck and Raper, 1925] and the conversion of the δ-keto-acids to succinic acid led Clutterbuck to suggest succinic acid as a possible intermediary between fat and carbohydrate.

The experiments now described show that under mild conditions of oxidation succinic acid may be formed in considerable quantity from oleic acid, and in the body this product would be further oxidised by the succinoxidase of the liver and muscles to malic acid.

A considerable body of evidence is accumulating that there is a widespread mechanism for dealing with the oxidation of succinic acid, not only in the animal body but also in lower organisms. Stent, Subramaniam and Walker [1929], for instance, have shown that *Aspergillus niger* contains enzymes by which succinic is converted through malic to oxaloacetic acid and point out that the conversion of the latter to pyruvic and lactic acids would form a bridge over which one might pass from fat to carbohydrate. They also found that when *A. niger* acts on butyric acid small amounts of succinic acid are formed; they appear to prefer the view that these are formed rather by dehydrogenation of acetic acid derived from the oxidation of the butyric acid than by direct oxidation of the butyric acid. We carried through an oxidation of acetic acid under conditions similar to those we used for the oxidation of oleic acid but were unable to extract any succinic acid from the product: therefore the succinic acid produced in our experiments is certainly not derived from acetic acid.

In all our experiments on the oxidation of oleic acid traces of acetone have been identified in the neutral volatile product by means of the sodium nitroprusside reaction, and in one case this has been definitely confirmed by the preparation of a crystalline derivative. It is possible that the source of the acetone might be the 4-keto-7-hydroxycaprylic acid, which might suffer the following degradation:

$$CH_3.CH(OH).CH_2.CH_2.CO.CH_2.CH_2.COOH \rightarrow CH_3.CO.CH_2.COOH \rightarrow CH_3.CO.CH_3;$$

or the caprylic acid residue might undergo oxidation, leaving a butyric acid residue which might then be oxidised further, or the acetone might be derived from the remaining part of the oleic molecule. In any case the amount formed is only just detectable and possibly represents the product of some alternative path of oxidation.

The evidence that β-oxidation of the lower fatty acids can take place in

the body is convincing, but it does not necessarily follow that this is the only method of oxidation of the saturated fatty acids nor that it is the method by which the unsaturated acids undergo oxidation. The classical feeding experiments of Knoop which have led to the general acceptance of the β-oxidation theory have the disadvantage that they had to be carried out on the phenyl derivatives of the acids. In this case it would be impossible for the terminal butyric residue to be converted to succinic acid, since it would be a benzyl and not a methyl group that would be available for oxidation. Obviously, considerable caution must be exercised in accepting conclusions based on acids in which the terminal methyl is replaced by a benzyl group.

It is possible that alternative paths of oxidation exist in the body, so that the acids may be broken down either by a process of β-oxidation leading to the production of acetoacetic acid, or by a process involving γ-oxidation such as is now shown to occur in the laboratory with caprylic acid leading to the formation of succinic acid.

It may be that a catalyst exists in the body playing the part of the cupric salt *in vitro*, and that it is the failure of this catalyst to function effectively in starvation and in diabetes which leads to the production of large quantities of the acetone bodies.

Perhaps the solution of that mysterious problem, the manner in which "fat burns in the fire of carbohydrate" is to be sought in the effect of a liver stored with glycogen upon the oxidative mechanism. It is not impossible that the determining factor as to whether β- or γ-oxidation may occur is to be looked for in the nature of the liver cells and that the particular condition of the cell may be connected with its glycogen content.

In any case the possible degradation of oleic acid through succinic rather than through β-hydroxybutyric acid finds some support from the results now brought forward.

We are now investigating the influence of the copper catalyst on the oxidation of the higher saturated fatty acids by means of hydrogen peroxide.

SUMMARY.

1. When the oxidation of oleic acid was carried out by hydrogen peroxide at 95° under the conditions used by Dakin in his experiments on the oxidation of the higher saturated fatty acids, a fraction, agreeing in composition with that of diketostearic acid, was formed, and evidence of the presence of an α-keto-derivative of nonoic or decoic acid was obtained. Some dihydroxystearic acid was also present.

2. The carbonic, acetic and formic acids produced corresponded to the complete oxidation of from 5 to 10 % of the total oleic acid. Increasing the proportion of hydrogen peroxide increased the proportion of dihydroxystearic acid present.

3. The addition of a small amount of cupric salt greatly increased the extent of the oxidation. At 95° with a large excess of hydrogen peroxide, more

than 70 % of the original oleic acid was completely broken down to carbonic, formic and acetic acids. Succinic and 4-keto-7-hydroxycaprylic acids were also identified.

4. When the oxidation in the presence of a cupric salt was carried out at 60°, the amounts of carbonic, formic and acetic acids produced corresponded to the complete oxidation of about 20 % of the total oleic acid. The product obtained in greatest amount was a derivative of caprylic acid to which the formula 4-keto-7-hydroxycaprylic acid has been assigned. A considerable proportion of succinic acid was also present. A small quantity of an oil, insoluble in water, consisted of a mixture of various oxygenated derivatives of stearic acid, the composition of which varied in different experiments.

5. Among the products of oxidation of caprylic acid were succinic acid and the same 4-keto-7-hydroxycaprylic acid as was previously obtained from oleic acid.

6. The bearing of these results on the theory of β-oxidation is discussed.

We gladly acknowledge grants received from the Department of Scientific and Industrial Research which have made this investigation possible. The micro-analyses were carried out in Dr Schoeller's laboratory.

REFERENCES.

Batelli and Stern (1911). *Biochem. Z.* **30**, 172.
Battie and Smedley-MacLean (1929). *Biochem. J.* **23**, 593.
Bloor (1924). *J. Biol. Chem.* **59**, 543.
—— (1926). *J. Biol. Chem.* **68**, 33.
—— (1927). *J. Biol. Chem.* **72**, 326.
Cahen and Hurtley (1917). *Biochem. J.* **11**, 164.
Clutterbuck (1927). *Biochem. J.* **21**, 512.
— and Raper (1925). *Biochem. J.* **19**, 385.
Dakin (1908). *J. Biol. Chem.* **4**, 227.
—— (1910). *Amer. Chem. J.* **44**, 41.
Edmed (1898). *J. Chem. Soc.* **73**, 627.
Ellis and Zeller (1930). *J. Biol. Chem.* **89**, 185.
Embden and Marx (1908). *Hofmeisters Beiträge*, **11**, 319.
Hazura (1888). *Monatsh.* **9**, 471.
Lapworth and Mottram (1925). *J. Chem. Soc.* **127**, 1628, 1986.
Leathes and Raper (1925). The fats. (Longmans Green and Co.)
Le Sueur (1901). *J. Chem. Soc.* **79**, 1313.
Morrison and Nash (1930). *J. Biol. Chem.* **88**, 479.
Pearson and Raper (1927). *Biochem. J.* **21**, 875.
Saytzeff (1886). *J. pr. Chem.* **33**, 300.
Spiridonoff (1887). *J. Russ. Chem. Soc.* **19**, 646.
Stent, Subramaniam and Walker (1929). *J. Chem. Soc.* 1987.
Terroine, Bonnet, Kopp and Véchot (1927). *Bull. Soc. Chim. Biol.* **9**, 604.
Thunberg (1909). *Skand. Arch. Physiol.* **22**, 430.

CXLI. SOME BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF COPPER IN ANIMAL NUTRITION.

By IRA JAMES CUNNINGHAM.

From the Rowett Research Institute.

(*Received June 26th, 1931.*)

WITHIN recent years our conception of the mineral nutrition of animals has had to be broadened to take account of certain "trace" elements which have been found to be widely distributed in the vegetable and animal kingdoms. These elements, *e.g.* manganese, iodine, zinc, copper, *etc.*, occur only in minute traces but their possible physiological significance cannot be neglected.

The claim that copper is essential for the utilisation of iron in haemoglobin building [Waddell *et al.*, 1928] arouses particular interest in this element. The work outlined below was undertaken with a view to obtaining further information on this and cognate subjects.

I. THE OCCURRENCE OF COPPER IN NATURE.

(*a*) *The vegetable kingdom.*

The occurrence of copper in vegetable materials has been investigated mainly with the object of discovering the amounts of this element in materials used as foodstuffs. Such investigations date as far back as 1816 [Meissner, 1816] but the greater number have been carried out within the last eleven years [Maquenne and Demoussy, 1920; Fleurent and Levi, 1920; Guérithault, 1920; Quartaroli, 1928; Elvehjem and Hart, 1929; Lindow, Elvehjem and Peterson, 1929, *etc.*]. The data available show that copper occurs very widely in foodstuffs, the amounts varying somewhere between 4 and 20 mg. per kg. dry matter, but the copper content may be affected by soil conditions [Webster and Jansma, 1929] and increased by manurial treatment of the crop [Elvehjem and Hart, 1929].

Maquenne and Demoussy [1920] have shown that, while copper occurs throughout all parts of the same plant, the concentration is greatest in buds, young leaves and cell sap. This has been confirmed to some extent by McHargue [1926], whilst Javillier and Guérithault [1927] found that the concentration of copper in the germs of seeds was much greater than in the endocarp.

Apart from these observations on the occurrence of copper in foodstuffs and on the concentration in young parts of plants, no evidence has been found of a definite function of copper in plant life.

In view of the connection, in animal life, between copper and the porphyrin pigment haemoglobin, it was decided to determine whether the copper content of different parts of plants bore any relation to the chlorophyll content. Comparison was therefore made of the copper contents of the root, the stem and the leaf of plants such as turnips and between the outer and the inner leaves of cabbages and lettuces. Results are shown in Table I. The second column refers to a second series of analyses.

The analytical method for copper used throughout the work reported in this paper was that of Biazzo [1926] as modified by Elvehjem and Hart [1929]. The only further modification employed was the addition of sodium phosphate to the solutions of the ashed materials in order to avoid interference by traces of iron.

Table I. *Comparison of the copper contents of aerial and root portions of plants.*

Plant	Part analysed	Mg. Cu/kg. dry matter	
Leek	Leaves	9·8	—
	White edible portion	5·8	—
Lettuce	Outer leaves	18·7	15·8
	Inner leaves	25·8	25·4
	Centre stalk	12·6	—
Cabbage	Outer green leaves	4·7	4·8
	Inner white leaves	4·0	5·8
	Centre vein of inner leaves	2·1	2·9
	Centre vein of outer leaves	3·1	
	Centre stalk of plant	3·4	—
Carrot	Leafy part (excluding lower leaves)	10·7	10·1
	Stalks of leaves	—	5·0
	Red part of root	4·5	5·4
	Yellow part of root	—	5·5
Beetroot	Leaves	10·2	12·6
	Stalks	6·8	8·4
	Roots	6·8	6·6
Potato	Stalk	11·2	—
	Tuber	5·4	—
Turnip	Leaves	7·1	—
	Stalks	3·0	—
	Roots	4·4	—
Garden pea	Mature old seed	7·3	—
	Young green seed	8·6	—
	Pods of young seed	5·4	—
	Leaves	10·6	—

		Mg. Cu/kg. dry matter
Average (excluding cabbage and lettuce):	Leaves	9·9
	Stalks	6·7
	Roots	5·5

It is seen that there is more copper in the aerial parts of plants than in the roots. This is probably not connected with chlorophyll content as the white inner leaves of cabbages and lettuces contained as much (or more) copper as the outer chlorophyll-rich leaves. It is notable that in stems and pods, where a large amount of fibrous material is to be found, the copper content is lower.

(*b*) *The animal kingdom.*

The wide distribution of copper in vegetable foodstuffs suggests its occurrence in animal organisms, and data are available showing the presence of this element in insects, marine animals and land animals.

(i) *Insects and marine animals.* Information concerning the copper content of insects is not very definite. Muttkowski [1921] obtained a positive qualitative test in samples representing 34 different species and Haber [1926] showed qualitatively that copper occurs in the blood of *Blatella germanica*. Quantitative figures are available only for three species [Giunti, 1879; Aronssohn, 1911].

In the case of marine animals, on the other hand, a considerable number of copper determinations have been published by Rose and Bodansky [1920] and by Severy [1922]. It was shown that of 35 species the copper contents varied from about 60 mg./kg. dry matter in the oyster to traces in the whale, the amounts diminishing progressively in ascending from the comparatively simple to the more complex organisms.

The presence of copper in marine animals has been explained only in the case of those animals whose blood contains haemocyanin.

Analytical data obtained by the writer for the copper contents of insects and of marine animals are given in Table II.

Table II. *Copper contents of some insects and marine animals.*

			Mg. Cu/kg. dry matter	Mg. Cu in whole organ or tissue of one animal
Insect		Cockroach (*Blatta orientalis*)	32·4	—
		House fly (*Musca domestica*)	77·6	—
		Honey bee (*Apis mellifica*)		
		Whole worker	25·0	—
		Fully developed queen bee grubs	24·5	—
		Young queen bee grubs	22·7	—
		Royal jelly	17·8	—
Marine animals		Octopus*		
		Skin	108·3	—
		Muscle	10·9	—
		Ink sac	914	8·8
		Ink (small sample)	No copper detectable	
	Tunicate	Mantle	10·9	0·020
	Lobster*	Liver	25·1	0·20
		Eggs	83·2	—
		Gonad	21·0	—
	Herring	Liver	14·2	0·008
		Skin	2·3	0·004
		Flesh	2·3	0·039
		Female reproductive organs	2·1	0·024
		Male „ „	2·3	0·022

* Samples washed with many changes of saline and finally with pure distilled water to free from traces of haemocyanin.

The figures for insects call for small comment except that it is of interest that the copper percentage of the queen bee grub is higher than that of royal jelly, its sole source of food.

Referring to marine animals, it is seen that there is a very high proportion and amount of copper in the ink sac of the octopus. Such accumulation of copper in the ink sac is worthy of note particularly as the ink contains no appreciable quantity of this element. Reference will be made later to observations of a catalytic influence of copper on the oxidation of *l*-3:4-dihydroxyphenylalanine to melanin. Since the ink of such sea animals contains melanin it is possible that, in the ink sac, copper has a function in the formation of the ink.

(ii) *Copper in land animals.* The occurrence of copper in land animals has been reported, but only in the case of the rat is there reliable information regarding the distribution of copper throughout the various organs of the body [Lindow, Peterson and Steenbock, 1929]. Some figures are available for different organs of man, the ox and the horse, but the data are scanty and the practice of taking different organs from separate animals has not permitted of definite conclusions being drawn regarding the distribution of copper throughout the body. The livers only, of a number of other species, have also been investigated for copper content.

In the present work, copper was determined in the organs from 12 different species of animals and the results are reported in Table III. The figures in a

Table III. *Copper in organs of different species expressed as mg./kg. dry matter.*

Species	Description	Liver	Heart	Lungs	Spleen	Kidney	Pancreas	Brain	Flesh	Skin	Hair
Human	Adult*	24·9	—	—	5·2	17·5	4·3	17·5	—	—	—
Bovine	Adult†	77·0	15·6	5·3	2·9	19·7	3·8	—	—	—	—
Bovine	Calf newly born	470·0	14·8	4·9	4·8	15·7	5·5	—	4·8	—	—
Bovine	Foetus	262·8	10·4	3·6	5·4	8·5	—	—	2·9	2·1	—
		(16·3)	(0·086)	(0·115)	(0·060)	(0·087)	—	—	—	(0·458)	—
Sheep	Adult	236·6	17·9	9·6	5·0	17·8	7·7	—	—	—	—
		(60·0)	(0·68)	(0·75)	(0·08)	(0·36)	(0·04)	—	—	—	—
Horse	Adult†	14·8	17·6	6·8	3·2	28·9	—	—	—	—	—
		(30·5)	(11·9)	(10·0)	(1·7)	(8·2)	—	—	—	—	—
Pig	Adult†	41·3	14·9	5·3	6·0	21·1	—	—	—	—	—
		(19·9)	(0·86)	(0·55)	(0·13)	(1·35)	—	—	—	—	—
Pig	Few days old	232·8	12·8	3·4	6·7	14·7	—	—	—	—	—
		(2·0)	(0·020)	(0·010)	(0·0013)	(0·050)	—	—	—	—	—
Dog	9 days old	98·2	17·4	6·2	—	14·2	—	8·5	—	9·9	22·7
		(0·320)	(0·006)	(0·005)	—	(0·009)	—	(0·012)	—	(0·076)	(0·056)
Cat	Adult†	25·3	14·4	3·8	5·2	10·1	—	14·6	2·3	4·2	11·9
		(0·534)	(0·039)	(0·033)	(0·016)	(0·075)	—	(0·079)	—	(0·433)	(0·790)
Guinea-pig	Adult	17·0	21·2	9·5	—	19·9	—	—	—	—	—
		(0·102)	(0·005)	(0·006)	—	(0·016)	—	—	—	—	—
Rabbit	Adult	9·2	22·3	8·1	—	13·7	—	—	—	—	—
		(0·17)	(0·023)	(0·018)	—	(0·040)	—	—	—	—	—
Rat	About 90g. wt.†	10·0	27·8	9·5	8·1	22·6	—	10·2	3·8	7·3	14·8
		(0·016)	(0·0023)	(0·0015)	(0·0016)	(0·005)	—	(0·003)	—	(0·025)	(0·014)
Badger	Adult†	21·7	12·8	5·6	3·0	9·4	—	10·8	—	3·2	24·7
		(2·09)	(0·112)	(0·100)	(0·019)	(0·094)	—	(0·089)	—	—	—
Domestic fowl	Adult†	12·4	14·9	2·4	—	11·7	—	—	—	Feathers	4·9
		(0·081)	(0·014)	(0·003)	—	(0·018)	—	—	—	—	—
Average:			16·8	6·0	5·0	16·4	—	—	—	—	—
Range of variation:			10·4–27·8	2·4–9·5	2·9–8·1	8·5–28·9	—	—	—	—	—

* Average of 3 series of analyses. † Average of 2 series of analyses.

horizontal line refer to the organs from one and the same animal or group of animals and the total amount of copper, expressed in mg. in the entire organ from one animal, is given in brackets. These figures may be used in discussion of the distribution of copper throughout the various organs of animals from any one species without encountering any effect due to individuality or diet.

The averages and ranges of variation of the copper content of heart, lungs, spleen and kidneys for all species are also given in Table III.

Although the ranges of variation for heart, lungs, spleen and kidneys seem rather wide it should be noted that the extreme figures occur in only a few cases, most of the figures falling within 50 % of the average. The differences in copper content between these different organs therefore appear to be real differences and not due to chance variation.

Further investigation was undertaken to find the variation of copper in any one organ due to individuality, under controlled conditions of feeding. In this case, copper was determined in the livers of normal newly born piglings, whose mothers had all been receiving the same diet. The averages and range of variation for 15 livers are given in Table IV and figures for iron content are also included.

Table IV. *Copper and iron content of infant pig livers.*

	Mg. per kg. dry matter		Mg. in whole liver	
	Copper	Iron	Copper	Iron
Average	241·4	1390	1·15	6·14
Range	50·8–465·7	580–1910	0·11–2·00	2·63–12·12

Again, although the ranges of variation are rather large, only in 4 of the 15 cases did the total copper in the liver vary more than 50 % from the average. The proportion of copper in the dry matter varied in a similar manner. There was no correlation between the iron and copper contents, but the variations in respect of iron were as great as those in respect of copper.

In all the organs so far dealt with, then, the variation of copper content was too great to assign a definite value for it in a given organ. At the same time the differences between the different organs were significant, and the distribution of copper throughout the organs was substantially the same for all species examined. Classification of organs according to descending value for either proportion of copper or total weight of copper in the organ gave liver, kidneys, heart, lungs, pancreas, spleen.

Analyses of organs not referred to in this list or in Table III are given in Table V. It is seen that copper is present in all the parts of animals (and of plants) examined, and it is possible that this element may fill a small corner in the composition of protoplasm. Fox and Ramage [1931] have made a similar suggestion from spectrographic examination of animal tissues.

A general division of the parts of animals may be made, giving two classes

(*a*) those containing a high proportion of copper;

(*b*) those containing a low proportion of copper.

Table V.

Species	Sample	Mg. Cu/kg. dry matter
Human	Hair	Av. 2 15·1
Bovine	Stomach wall	Av. 3 6·5
	Parotid salivary gland	Av. 2 6·6
	Horn	3·9
	Hoof	2·1
	Fatty bone marrow	0·16
	Red bone marrow, calf	Av. 2 4·4
	Thymus, calf	3·5
Sheep	Thymus	6·5
	Lymph glands	10·8
	Digestive tract	9·0
Horse	Adrenal	18·0
Dog	Melanotic tumour	Av. 2 8·0
	Bile	71·0
Cat	Bile	Av. 2 22·7
Rat	Tongues	Av. 2 16·6
Domestic Fowl	Skins and scales of leg	4·7
	Proventriculus	12·8
	Gizzard	3·2
	Gizzard lining	Av. 2 27·0

In (*a*) are included liver, kidneys, heart, brain, hair, while (*b*) includes skin, lungs, pancreas, spleen and flesh.

It was thought possible that some function might be associated with the presence of copper in certain organs and tissues, and further investigations were made of the skin and hair and the liver.

Skin and hair. Appreciably high amounts of copper were found in the skin and hair of all animals examined. Some figures were given in Table III and further figures are given in Table VI.

Table VI. *Copper in skin and hair of animals. Mg. Cu/kg. dry matter.*

Species		Skin	Hair or Wool
Bovine	Epidermis	6·0	
	Dermis	2·3 (av. of 2)	
Sheep		2·8 (av. of 3)	8·3 (av. of 10)
Rabbit		6·6 (av. of 6)	15·4 (av. of 4)
Rat		9·7 (av. of 2)	12·2 (av. of 2)
Rats from same litters	At birth	18·6 (av. of 4) (0·0020)*	
	1 week old	14·8 (av. of 4) (0·0090)	
	2 weeks old	9·3 (av. of 4) (0·0105)	
	3 weeks old	13·7 (av. of 2) (0·036)	
Guinea-pigs from same litter	At birth	21·4 (0·070)	12·5 (0·029)
	1 week old	10·1 (0·035)	13·7 (0·031)
	2 weeks old	11·0 (0·036)	11·8 (0·034)

* Figures in brackets represent the weight in mg. of copper in the whole tissue or part from one animal.

The figures quoted indicate that, in rats, the amount of copper in the skin increased rapidly from birth and reached a fairly steady value at about three

weeks of age, while in guinea-pigs the amount of copper decreased from the value at birth and became constant at about one week old. Several other series of analyses were made of both these species in which the age limits were narrowed or lengthened, and the findings from these data, which for the sake of brevity are not cited here, were in complete accord with the typical results quoted in Table VI.

The presence of copper in rat skins in somewhat different amounts has previously been reported by Lindow, Peterson and Steenbock [1929]. These workers found that the average amount of copper in the skin and hair of an adult rat was 0·161 mg. and the proportionate amount was 3·57 mg. copper/kg. dry matter. According to them, feeding a high level of copper to rats for 7 weeks did not increase the copper content of the skins above this value.

The author has investigated the effect of the amount of copper in the diet on the copper content of rat skins with the following results.

Table VII. *Copper content of rat skins (average values).*

			Skin		Hair		Skin and Hair	
No. of rats	Copper in diet mg. daily	Time fed diet months	mg. Cu per skin	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter
4	0·011	9	0·054	6·9	0·022	9·5	0·076	8·1
3	0·050	9	0·048	6·9	0·021	13·8	0·069	8·2
3	0·075	7	—	—	—	—	0·064	4·4
1	7·5	1	—	—	—	—	0·078	—
3	7·5	7	—	—	—	—	0·113	6·8

A slight increase in the total copper content of the skin and hair occurs after prolonged feeding of high doses of copper, but the effect is not very marked. At the same time almost identical amounts of copper were found in the skins of rats fed such different levels of copper as 0·011 and 0·050 mg. daily for long periods. It is therefore unlikely that the skin is a path for excretion of copper.

The occurrence of somewhat uniform amounts of copper in the skin and hair of many species merited some investigation. No explanation can be offered in the case of the hair, though it is of interest to note (1) that in bovines the concentration of copper is greater in the epidermis than in the dermis (Table VI), and (2) that in the sheep, an animal which produces large amounts of wool, the copper content of the liver is much higher than in any other species. On the other hand, while Angora rabbits produce large amounts of hair, the copper content of the livers is of the same order as that of short-haired rabbits.

Some instances were noticed amongst rats and rabbits in which the copper content of the skins of black animals was higher than for white animals. While this was not always so, the relation of copper to pigmentary processes was considered and the effect of this element on the oxidation of "dopa" (*l*-3:4-dihydroxyphenylalanine) was tried. "Dopa" is a chemical precursor of melanin into which it is converted on oxidation. It has been used by Bloch [1927] and by Stewart and Percival [1930] as a histological stain for detecting "dopa"

oxidase in skin, and by Koller [1930] in studying the genetics of pigmentation in rabbits. The technique adopted was largely based on the work of Koller.

It was found that the oxidation of a solution of "dopa" by means of an extract of the skin of newly born rats, rabbits or guinea-pigs only proceeded slowly, maximum coloration at room temperature occurring after about 60 hours. The addition of copper to the solution materially accelerated the oxidation process, always in proportion to the amount of copper added, and the addition of 0·05 mg. of copper in 2 cc. of solution sufficed to give maximum coloration in 18 hours.

It is difficult to assess the significance of this catalytic action of copper, but it is of interest to consider the possibility already alluded to that the copper in the octopus ink sac may possibly be definitely related to the formation of the melanin pigment present in the ink. It is possible also, that in land animals the copper in the skin may play some similar part in the formation of melanin pigments.

A further connection between copper and pigmentation is to be found in the practices of bird fanciers for obtaining a deep yellow colour in canaries, in which copper sulphate, or other substances such as cayenne pepper, are fed to moulting birds. The new feathers are said to have uniformly deep yellow coloured edges and this was confirmed experimentally.

This effect of copper feeding is probably not due to an increased formation of a copper pigment such as turacin, the pigment of Turaco feathers [Church, 1892], since the copper content of canary feathers was found to be low compared with the content of turacin-bearing feathers. Analytical results are given in Table VIII.

Table VIII. *Copper content of feathers.*

	Mg. Cu/kg. dry matter
Canary (average of 3)	40
Turacus Hartlaubi medius	
Crimson, turacin-bearing, secondary wing feathers	2900
Black wing feathers (no turacin)	14

Thus, although there is a possibility that the copper in the skin may exercise some influence on pigmentary processes, evidence bearing on the subject is too scanty for any conclusions to be drawn.

Copper in liver. In every species of land animal studied the liver was found to contain a much greater amount of copper than any other organ. The ratio, weight of copper in liver to weight of copper in kidneys, was found to be, for the sheep 160, the horse 4, the cat 6, the rabbit 40, and of similar order for the other species. This observation also applies to such figures as have been published by other workers.

A notable feature is the high proportion of copper in sheep's liver; the average copper content of 9 livers was 323 mg. copper per kg. dry matter, the range being 212·5–497·5. Since there is in this country no well-established practice of dosing sheep with copper sulphate for internal parasites, this high

copper content cannot be due to such administration of copper. Figures of the same order have been reported by Aston [1911] for livers of normal New Zealand sheep.

The high proportion of copper in livers generally might be due to filtration, from the blood, of copper absorbed in the digestion of foodstuffs. In this connection, figures published by Lindow, Peterson and Steenbock [1929] showed that feeding rats 5 mg. copper (as $CuSO_4$, $5H_2O$) per rat daily for 7 weeks increased the copper content of the livers of the rats nearly 20 times. The author has fed different amounts of copper to rats with the results given in Table IX.

Table IX. *Copper content of rat livers.* (*Average values.*)

Diet	Copper in diet mg. daily	Time fed diet months	No. of rats	Mg. Cu in whole liver	Mg. Cu/kg. dry matter
Stock	about 0·050	Extended period	21	0·020	12·8
Cow's milk only	—	,,	10	0·010	14·1
Synthetic	0·011	9	1	0·015	10·0
,,	0·050	9	3	0·023	13·9
,,	7·5	4	1	0·036	41·7
,,	7·5	5	8	0·049	19·6

The copper content of rat livers, then, depended on the amount of copper in the food but the storage observed when high proportions of copper were fed was markedly less than was observed by Lindow, Peterson and Steenbock [1929].

Storage of copper in livers of foetal animals. That a considerable storage of copper occurs in intra-uterine life has previously been shown in the case of the human [Morrison and Nash, 1930], bovine [McHargue, 1925, 1, 2] and porcine species [Powick and Hoagland, 1924; Hart *et al.*, 1930]. Similar observations have been made by the writer for a number of species and the results are collected in Table X.

Table X. *Copper in livers of adult, foetal and newly born animals.* (*Average values.*)

Figures in brackets give the number of analyses.

Species	Mg. Cu/kg. dry matter: Adult	Foetal	Newly born
Human	25 (3)	281 (1)	—
Bovine	70 (3)	171 (4)	550 (2)
Sheep	323 (9)	—	117 (3)
Pig	35 (7)	—	241 (15)
Guinea-pig	17 (3)	122 (3)	203 (4)
Rabbit	10 (7)	29 (3)	55 (4)
Rat	12·5 (23)	—	70 (3)
Dog	88 (3)	—	89 (2)

In the bovine, rabbit and guinea-pig species, where comparisons of the copper contents of livers of foetal and newly born animals were made, it was found that there was more copper in the liver of the newly born animal than in that of the foetal animal.

With the exception of sheep, and possibly dogs, the figures for the different species show a much higher percentage of copper in the liver of the newly born or foetal than in that of the adult animal. In all cases, even with lambs and puppies, it was found that there was a steady drop in the percentage of copper in the liver from birth onwards, throughout early infancy (Table XI).

Table XI. *Copper content of infant livers at different ages.*

Figures in brackets give the mg. Cu in the whole liver.

	Mg. Cu/kg. dry matter							
Age / *Species*	At birth	3 days	1 week	2 weeks	3 weeks	4 weeks	8 weeks	12 weeks
Sheep	117·0	67·2	—	—	—	14·5	—	—
Dogs (same litter)	—	—	69·2 (0·209)	—	—	—	45·3 (0·534)	13·7 (0·287)
Rats (same litter)	87·8 (0·0045)	—	23·5 (0·0050)	14·5 (0·0063)	17·8 (0·022)	—	—	—
Guinea-pigs (same litter)	149·4 (0·171)		59·6 (0·102)	30·8 (0·042)	—	—	—	—

The observations recorded show that, of all organs the adult liver contains the highest proportion and absolute amount of copper, that the copper content of the diet influences the amount of copper in the adult liver, but not to a marked degree, and that the livers of newly born animals are provided with a large store of copper. This storage is possibly to provide the young animal with a supply of copper over the suckling period when only insignificant amounts of this element would be available in the diet of milk.

II. Copper and iron metabolism.

Copper and haemoglobin building.

As has been mentioned, one of the chief interests of copper arises from the claim by Hart *et al.* [1928] that, in the presence of this element inorganic iron may be used by rats for haemoglobin building.

Bunge [1902] stated that only "food" iron, *i.e.* iron in firm combination with organic compounds, as in haematogen, could be used by animals for haemoglobin building, and that the iron of iron salts or "inorganic" iron could not be used for this purpose. Even at the present day opinion is divided on this point and experimental evidence has been conflicting. However, the value of iron therapy in all types of human anaemias is well known and the success of this practice in curing Bush Sickness in New Zealand [Aston, 1921] and anaemia in young pigs in Scotland [McGowan, 1924] renewed interest in iron metabolism and particularly in haemoglobin production.

Waddell *et al.* [1928] have shown that newly weaned rats became anaemic in 4–6 weeks when their diets were restricted to cow's whole milk, and that the anaemia was not cured by supplementary feeding of purified "inorganic" iron. When copper was fed along with the "inorganic" iron, however, a rapid cure was effected. McHargue, Healy and Hill [1928], McCollum and Becker [1930],

Weichselbaum [1930] and Krauss [1930] have confirmed this effect of copper, but, on the other hand, Beard and Myers [1929] and Keil and Nelson [1931] found that purified "inorganic" iron alone would slowly cure the anaemia produced in rats by milk feeding. It has further been claimed by Titus and Hughes [1929] that manganese is as effective as copper in bringing about a cure of such an anaemia, and Beard and Myers [1929] also found that nickel, cobalt and germanium were as good supplements as copper. Mitchell and Miller [1929] obtained a maximal regeneration of haemoglobin when the ash of spinach was fed with a milk-iron diet and, since spinach ash contains many "trace" elements, these authors postulated that a complex of "trace" elements must be present in the diet along with iron for optimal utilisation of the last named element in haemoglobin building. Nevertheless, Weichselbaum [1930] obtained no haemoglobin regeneration when iron *plus* manganese or iron *plus* cobalt was fed to rats, and Krauss [1931] found no better effect with iron *plus* copper *plus* manganese than with iron *plus* copper. Again Waddell, Steenbock and Hart [1929] reported no effect with the twelve "trace" elements zinc, chromium, germanium, nickel, cobalt, lead, antimony, cadmium, tin, mercury, arsenic and manganese, fed singly or all together as supplements to a milk-iron diet.

All the work cited above has been concerned with the supply to, and utilisation of iron by, rats fed a basal diet of milk. Drabkin and Waggoner [1930] studied the effect of protein on haemoglobin regeneration. They found that rats made anaemic with milk diets were brought to normal with great rapidity when the diet was changed to a solid one containing iron but practically devoid of copper. Drabkin and Miller [1931] observed relief from anaemia, when arginine or glutamic acid was used as a supplement to the milk diet, while no success was recorded from feeding histidine. These authors believed that the anaemia produced by milk feeding was not due to a deficiency of iron but to deficiency of the pyrrole nuclei necessary in formation of the porphyrin moiety of haemoglobin.

From this brief survey it is seen that, while the effect of copper in promoting the utilisation of iron in haemoglobin regeneration has received considerable support, it has not been thoroughly established that copper is essential. A study has therefore been made of haemoglobin building from "inorganic" iron, alone or with various supplements.

Rats were used throughout the investigations and the method first employed, due to Waddell *et al.* [1928] was as follows. Anaemia was produced by feeding newly-weaned animals a diet consisting solely of cow's whole milk, and supplements were then fed individually to the rats and their effects measured by response in haemoglobin content of the blood.

Haemoglobin was determined with the Haldane haemoglobinometer, and on this scale the normal haemoglobin content of rat's blood is 80–90 %. Blood samples were obtained by puncturing a coccygeal artery or vein on the ventral aspect of the tail. The advantage of this procedure over the usual one of

snipping a small piece from the end of the tail is that the flow of blood can be regulated according to the condition of the animal by making a puncture near the base or near the tip of the tail.

The conditions of experimentation demanded rigid exclusion of all possible sources of metallic contamination, and special cages were therefore constructed from glass battery jars 13″ × 12″ × 9″ high. A false bottom of perforated celluloid allowed drainage of urine, whilst sufficient ventilation was provided by perforating the celluloid plate which closed the front of the cage. The milk used in feeding was drawn directly from the cow into an earthenware jar and the rats were fed from glass vessels.

It was found that deaths occurred very early when newly weaned rats were fed on the diet of cow's milk. In a number of experiments, 130 rats were used and of these 64 died after 2–5 weeks' milk feeding, the average time of survival being 3·2 weeks. After about 5 weeks' feeding the blood of the remaining animals contained low amounts of haemoglobin and these were given supplements. The iron used was purified by treatment in acid solution with H_2S, followed by several precipitations from acid solution by ammonia. For feeding purposes it was nearly neutralised with pure NaOH and for injection purposes the osmotic pressure was adjusted with pure NaCl. The response in haemoglobin content of the blood is shown in Fig. 1.

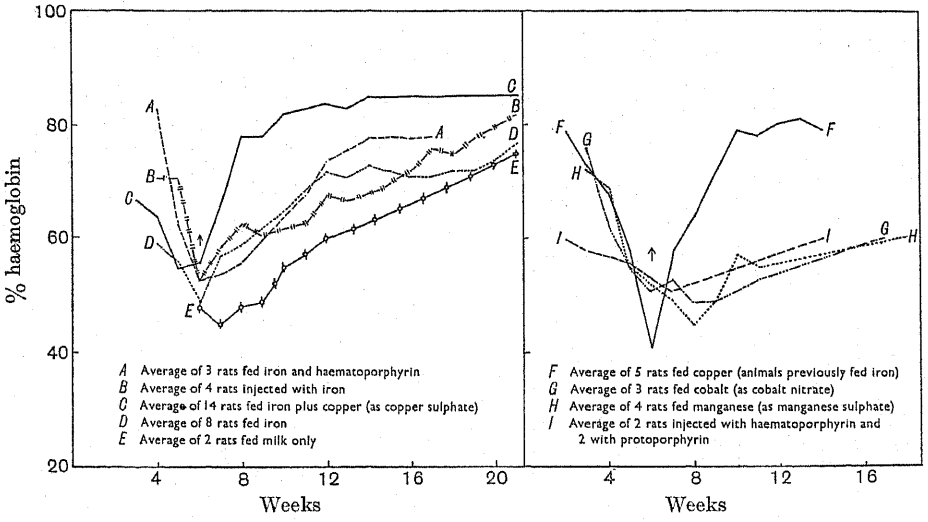


Fig. 1. Haemoglobin curves of rats fed milk diets from 0 weeks with various supplements from ↑.

The effect of copper feeding is at once obvious, for the rate of haemoglobin regeneration in rats fed this element is much more rapid than that observed when any other supplements were used. Curve *F* is of special interest. The animals to which it refers had previously been cured of anaemia by prolonged feeding of iron supplements and it was to be expected that considerable

quantities of iron would be stored in the liver. This was shown by comparing the iron contents of the livers of rats treated similarly up to the point marked ↑ on the curve, with those of rats fed milk alone. The following average figures were obtained.

	Mg. Fe in whole liver	% Fe in dry matter
Rats fed milk *plus* iron (long period)	2·493	0·158
Rats fed milk alone	0·917	0·078

The rats became anaemic when iron supplements were stopped notwithstanding this store of iron. When copper alone was fed, a rapid regeneration of haemoglobin followed, and it is therefore probable that the copper feeding caused the utilisation of the stored iron. After some time the animals were killed, and the iron content of the livers was determined. An average figure of 1·183 mg. Fe per liver or 0·127 % was found. The reduced quantity of iron supports the suggestion that copper feeding caused utilisation of the stored iron.

In view of these results it would appear that:

1. Newly weaned rats when fed a diet consisting solely of cow's whole milk developed a condition of anaemia very rapidly.

2. While a large proportion of the rats died if the diet was continued, slow recovery occurred in a small number of cases.

3. Additions of inorganic iron and copper to the diet prevented death and markedly hastened the recovery from anaemia.

4. Injection of iron, protoporphyrin or haematoporphyrin prevented death but had very little effect on the time of recovery.

5. Supplementary feeding of iron, manganese, cobalt or haematoporphyrin prevented death but had very little effect on the time of recovery.

These conclusions must, however, be considered in the light of certain complicating factors. Thus, the death rate was very high and, to investigate this further, 34 rats were fed a diet of cow's milk, and haemoglobin estimations made weekly from the commencement of feeding, for a period of 27 weeks. The results obtained may be arranged in three groups.

(*a*) Those rats which died soon after the milk diet was commenced and the haemoglobin of which was low. 8 deaths of this type occurred.

(*b*) Those rats which died after a longer period of milk feeding, and in which no marked lowering of haemoglobin occurred. 14 deaths of this type occurred.

(*c*) 12 rats which lived throughout the experiment exhibiting no dangerous lowering of haemoglobin.

In Fig. 2 are plotted haemoglobin curves: rat 628 typical of (*a*), rat 624 typical of (*b*) and the average of the 12 rats in (*c*).

The results indicate that there is some doubt as to whether a true nutritional anaemia was developed in rats fed on a diet of cow's milk. On the one one hand the long period of survival of the 12 rats in (*c*) and, on the other hand, the large percentage of early deaths both in (*a*) and in earlier experiments suggest the possibility that some type of epidemic caused these deaths rather

than that they were due to an uncomplicated condition of iron starvation. Pathological examination of some of the anaemic rats from the earlier experiments was kindly made by Dr J. P. McGowan. The blood contained low

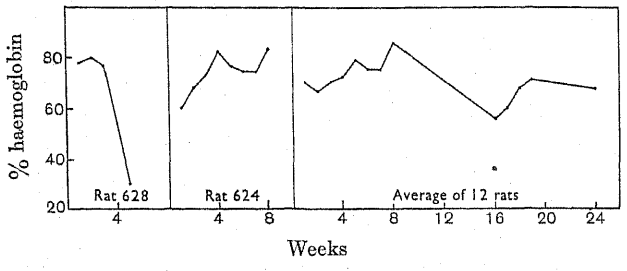


Fig. 2.

percentages of haemoglobin, but the bone marrow was hyperplastic, and marked reticulocytosis occurred in all cases. These conditions do not correspond with those of aplastic anaemias such as occur in iron deficiency and rather suggest an anaemia having as its characteristic feature a hyperplastic condition of the bone marrow. My attention was then drawn by Dr McGowan to the prevalence in rats of infections with Gärtner's bacillus and *Bartonella muris*.

Many of the animals which died in an acutely anaemic condition had *post mortem* symptoms corresponding to those in cases of death from *B. gärtner* [Boycott, 1911] and this organism was isolated[1] from the intestine of one of the animals.

Infection by *Bartonella muris* is thought to be latent in most rats, though the organism does not usually develop until the spleen is extirpated [Mayer, Borchardt and Kikuth, 1927]. Acute anaemia then results in a large proportion of the animals. Intact rats fed diets deficient in vitamins A and C may develop the disease [Wells and Mehta, 1930], and Meyer [1929] has shown that rats 3–4 weeks old are at the most susceptible stage and may be infected even when the diet is complete. He also found that the presence of *B. gärtner* sensitised some animals in a manner similar to splenectomy.

Newly-weaned rats (3–4 weeks old), restricted to a diet of cow's milk, consume only about 30 cc. daily, and thus receive only about 4 g. solid material per day compared with 10–15 g. consumed by rats on solid diets. The inadequate caloric value of the food coupled with the susceptible age of the rats [Meyer, 1929] give a set of conditions favourable to development of *Bartonella muris*.

It is likely, then, that the anaemia developed by milk feeding was partly due to *Bartonella* infection and such a possibility must be considered in other work, where milk diets have been employed for the production of anaemia. While it has been clearly demonstrated that copper feeding caused a more rapid regeneration of haemoglobin than did the feeding of iron alone, it was

[1] By kindness of Bacteriology Dept., Edinburgh University.

important to find if copper could exert any effect on haemoglobin production when the possibility of infection by *Bartonella muris* or *B. gärtner* was excluded.

A solid diet low in iron and copper was required, but the difficulty of compounding such a diet is illustrated by the results of analyses of foodstuffs (Table XII) likely to be free from these elements.

Table XII. *Iron and copper contents of some foodstuffs.*

Copper reported as mg. per kg. sample as purchased.
Iron reported as mg. per 100 g. sample as purchased.

Material	Copper	Iron	Material	Copper	Iron
Maize starch	0·8–1·9	3·18	"Casein" B.D.H. fat and vit.-free	3·9	—
Rice starch	0·8	2·02	Cheese (Scottish)	2·0	—
Potato starch	1·8	1·10	Cheese (Kraft)	0·45	1·23
Wheaten starch	2·4	0·7	Gelatin	9·6	—
Dextrin	2·2	—	Egg-albumin B.D.H.	1·4	1·00
Sago	Traces (0·1)	0·52	Egg-albumin (Chinese)	1·0	0·72
Castor sugar (sucrose)	Traces (0·1)	0·13	Butter (Scottish)	0·6	0·14
Arrowroot biscuits	1·5	1·03	Milk (Rowett Institute)	—	0·065 mg./100 cc.

By a selection from these materials it was, however, possible to devise a complete diet sufficiently low in iron and copper for use in an attempt to produce anaemia in growing rats.

Three groups of 6 rats were used in an experiment and the basal diet was as follows:

	Weight for 6 rats daily
Kraft cheese	3·0 g.
Egg-albumin	9·5 g.
Butter	9·5 g.
Sago	16·0 g.
Castor sugar	16·0 g.
Salt mixture	2·5 g.
Milk	60 cc.
Radiostol	1 drop

The salt mixture was McCollum's No. 185 modified by exclusion of ferric citrate and made up commercially from A.R. chemicals. Slight contamination had apparently occurred in commercial mixing since the salt mixture contained 2·2 mg. Fe per 100 g. Two further salt mixtures were prepared in the laboratory by the addition of purified "inorganic" iron to one and purified iron and copper sulphate to the other. These salt mixtures were used in the diets of two further groups of rats. The following amounts of iron and copper were supplied to the rats of the different groups.

	Intake per rat daily	
Group	Mg. iron	Mg. copper
I	0·058	0·006
II	0·35	0·006
III	0·35	0·046

The experiment lasted 18 weeks, haemoglobin determinations and reticulocyte counts being made at weekly intervals. The average haemoglobin values for the different groups are plotted in Fig. 3, while in all cases the percentage of reticulocytes was within the normal value of 2 % for the rat [Krumbhaar,

1930]. This normal value is in distinct contrast to the marked reticulocytosis of rats made anaemic by milk feeding and it is therefore unlikely that there was any complication from the infections referred to earlier.

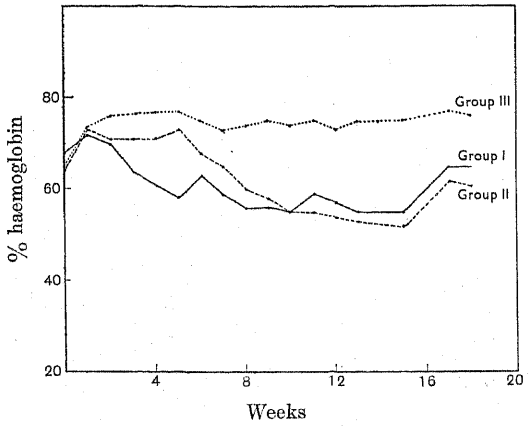


Fig. 3.

The results (Fig. 3) show that rats receiving no additions of iron or copper developed a mild anaemia which tended to disappear towards the end of the experiment. Supplementary feeding of inorganic iron had no effect in preventing the onset of this anaemia, but when copper was fed as well as iron the haemoglobin content remained normal throughout the entire period of the experiment.

Consideration of the weight increases shows that the effect of the copper is even more marked than is evident from the haemoglobin curves, for average growth in Group III was 76 g. while that in Group II was 57 g. and in Group I 55 g.

The additional copper, therefore, brought about the utilisation of iron for haemoglobin building, and inorganic iron was not used for this purpose unless copper also was available.

Other relations between copper and iron metabolism.

In the first experiment in this connection, 4 rats which had been fed for 3 weeks after weaning on a diet of starch and cow's whole milk were housed individually in metabolism cages. Faeces and urine were collected separately and 5 days' excretion from each rat was made into one sample and analysed for copper. After two initial periods on a basal diet low in iron and copper, inorganic iron was fed daily to each rat. The effect of this iron feeding on copper excretion was determined.

To avoid metallic contamination, metabolism cages were constructed from 8-inch Büchner funnels. The animals were kept on the perforated plate and, to provide sufficient height, the sides were lengthened by perforated sheet zinc

wrapped round the outside where it was out of contact with the rat or with excreta. A glass plate covered the top of the cage. Faeces remained on the perforated plate of the Büchner funnel and were removed daily, urine passed through and was collected in a beaker placed beneath. Cages were washed out at end of each five-day period. The analytical results are given in Table XIII.

Table XIII.

Average of 4 rats.

Date	Diet	Mg. Cu in Faeces	Mg. Cu in Urine	Cu in faeces / Cu in urine
10–15. ii. 30	Basal	0·028	0·007	4·0
16–20. ii. 30		0·021	0·010	2·1
21–25. ii. 30	Basal + 0·5 mg. Fe per rat daily	0·018	0·004	4·5
26. ii. 30–2. iii. 30		0·017	0·007	2·4
3–7. iii. 30		0·018	0·007	2·6
8–12. iii. 30	Basal + 2·0 mg. Fe per rat daily	0·017	0·006	2·8
13–17. iii. 30		0·016	0·007	2·3
18–22. iii. 30	Basal	0·016	0·006	2·6
23–27. iii. 30		0·018	0·006	3·0
29. iii. 30–1. iv. 30		0·017	0·006	2·8

Before being used for experiment the rats were kept for three weeks from weaning time on a diet low in copper. This precaution was adopted so that stored copper obtained from sharing the mother's food would be eliminated. The results for the first period are probably affected by incomplete preparation of the rats. Omitting the first period from consideration it can be concluded that feeding inorganic iron did not alter the total amount of copper excreted or the proportion appearing in faeces and urine.

The average ratio $\frac{\text{copper in faeces}}{\text{copper in urine}}$ is 2·8 for a diet low in copper.

In another experiment two groups of rats were housed in glass cages similar to those already described and fed a basal ration of cow's whole milk *ad lib. plus* 3 g. sucrose per rat daily. One group was fed in addition 0·2 mg. purified inorganic iron per rat daily. At the end of 6 weeks, the animals were killed and copper determined in liver, heart, lungs, spleen, kidneys, brain, flesh, skin, tongue and testes. The data for this experiment are averaged in Table XIV.

Table XIV.

	Basal diet 11 rats	Basal diet + iron 10 rats
Average gain in weight during experiment	36 g.	37 g.
Total copper in parts analysed (calculated for one rat)	0·037 mg.	0·038 mg.

No significant differences between animals of the two groups were found in any of the organs or parts analysed. The results therefore indicate that addition of inorganic iron to a diet of milk *plus* sucrose has no effect on the copper content of the rat.

An experiment was designed to study the effect of feeding a diet almost free from copper on storage of iron in the foetus. Work which is discussed below (see Table XIX) showed that the iron content of rat pups from mothers

receiving a low ration of copper was slightly, though not significantly, less than that of pups from mothers receiving a normal ration of copper. That complete removal of copper from the diet cannot be effected by selection of foodstuffs is illustrated in Table XII. In the case of the rat, storage in the liver takes place after continued ingestion of even small traces of copper (Table IX), and between pregnancies the adult maternal animal is probably able to store sufficient copper to supply the needs of the foetal and suckling young. On the other hand, the hen produces an egg at regular short intervals and is therefore constantly depleting iron and copper reserves. It was decided, therefore, to use the hen for this experiment.

Four groups of 5 birds were used, the feeding being as follows:

Group I	Basal ration + iron	Capons
II	Basal ration + iron	Pullets
III	Basal ration + iron + copper	Capons
IV	Basal ration + iron + copper	Pullets

The basal ration was as follows, the daily amounts for 5 hens being given:

Kraft cheese	$\frac{1}{4}$ lb.
Purified maize starch	$\frac{1}{4}$ lb.
Castor sugar	$\frac{1}{4}$ lb.
Salt mixture	10 g.
Radiostol	5 drops

thoroughly mixed and fed without further treatment.

Also

Whole milk	1 pint
Granite grit	*ad lib.* (purest form of grit available)
Glass-distilled water	*ad lib.*
Granulated pure $CaCO_3$	*ad lib.*

Precipitated $CaCO_3$ was granulated by mixing with paste prepared from starch and drying at 100°.

Salt mixture I	Groups I and II	
	McCollum's salt mixture 185	21 parts
	Pure $CaCO_3$	9 parts
	Purified iron	0·03 part
Salt mixture II	Groups III and IV	
	0·003 part copper (as $CuSO_4, 5H_2O$) were added.	

In a preliminary experiment the diet for Groups I and II was shown to maintain weight and laying in a group of 5 hens over a period of 1 month.

The birds were confined inside on wooden floors covered with sawdust. Food was placed in wooden troughs and liquids in glass vessels. Trap nests were provided to obtain individual laying records. Feeding was started about one month before laying was expected to begin in order that first formation of eggs in the ovary should take place while the experimental diet was being consumed.

In all except two cases the experiment had been in progress for about 3 months before laying commenced.

Since reproduction makes a strong call on all reserves of the mother it was thought that the iron store in the liver might be depleted to fulfil the iron requirements of the egg. Two groups of capons were therefore included to act as controls for iron content of liver at the end of the experiment. These capons were procured from the same hatching of chickens as the pullets. The use of capons as non-laying controls for hens receives some justification from the facts of sex reversion in the fowl.

Results. Growth was similar in all groups and was rather slower than for normally fed hens. No significant differences in haemoglobin content of the blood occurred between any of the groups. When the experiment was concluded, 71 eggs had been laid by Group II hens and 69 by Group IV hens.

Iron estimations were made on the contents of all the eggs, composite samples being made from each hen with 2–4 eggs per sample. No significant differences were recorded and the averages of all eggs from each group are therefore given. Average analytical figures for twelve eggs from hens hatched at the same time as the experimental ones but fed a practical ration are included for comparison. Copper analyses are also included. These results appear in Table XV. In Table XVI are given the group averages of the results of analyses for iron and copper in the livers of all the birds at the end of the experiment.

Table XV. *Iron and copper content of eggs (averages).*

	% dry matter	Mg. Fe per egg	Mg. Cu per egg	% Fe on dry matter	Mg. Cu/kg. dry matter
Group II. Low copper	25·03	0·897	0·021	0·0087	2·0
Group IV. Fed copper	25·06	0·940	0·021	0·0085	1·7
Normal stock feeding	25·28	0·990	0·030	0·0090	2·2

Table XVI. *Iron and copper content of livers (averages).*

	Mg. Fe per liver	Mg. Cu per liver	% Fe on dry matter	Mg. Cu/kg. dry matter
Group I	18·64 Range 7·00–38·50	0·061	0·206	6·3
Group II	11·60 Range 8·50–16·58	0·057	0·110	5·7
Group III fed copper	9·87 Range 5·40–12·83	0·085	0·088	7·5
Group IV fed copper	14·05 Range 9·40–23·75	0·057	0·118	5·0

No significant differences were found between the iron and copper contents of eggs from Group II hens (each receiving about 0·07 mg. copper per day) and Group IV hens (each receiving 0·27 mg. copper per day). The amounts of iron in the livers of capons and of hens fed the experimental ration varied too much for any conclusions to be drawn.

Further experiments were conducted with rats to determine the effect of

copper feeding on the iron content of the livers and bodies of rats. The basal diet was cow's whole milk *ad lib. plus* 3 g. sucrose per rat daily, and the groups were treated as follows:

Group I Killed at weaning and analysed.
II Fed basal ration from weaning.
III Fed basal ration from weaning *plus* 0·2 mg. iron/rat daily.
IV Fed basal ration from weaning *plus* 0·2 mg. iron *plus* 0·05 mg. copper/rat daily.
V Fed basal ration from weaning *plus* 0·05 mg. copper per rat daily.

Feeding was continued for one month, after which the rats were killed and the iron determined in the liver, spleen, and rest of the rat (intestinal tract excluded). These conditions apply to the first two experiments; in the third experiment Group I was omitted and, in analysis, the spleen was included with the rest of the rat.

The average analytical results for each experiment are given in Table XVII.

Table XVII.

	Killed at weaning Group I		Basal diet Group II		Basal diet + Fe Group III		Basal + Fe + Cu Group IV		Basal + Cu Group V	
	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter
Exp. 1.	4 rats		2 rats		3 rats		2 rats		2 rats	
Liver	0·308	0·038	0·498	0·047	1·149	0·115	0·764	0·069	0·351	0·032
Spleen	0·066	0·090	0·119	0·090	0·251	0·166	0·272	0·195	0·185	0·109
Rest of rat	1·10	0·012	1·31	0·011	2·24	0·014	2·49	0·015	2·02	0·011
Exp. 2	4 rats		3 rats		4 rats		2 rats		3 rats	
Liver	0·249	0·053	0·302	0·037	0·716	0·079	0·845	0·086	0·320	0·038
Spleen	0·024	0·101	0·092	0·056	0·117	0·108	0·157	0·110	0·100	0·073
Rest of rat	0·89	0·015	1·01	0·007	1·65	0·010	1·79	0·017	1·30	0·011
Exp. 3			7 rats		4 rats		5 rats		3 rats	
Liver	—	—	0·615	0·056	0·663	0·046	0·650	0·055	0·597	0·043
Rest of rat	—	—	1·46	0·012	1·91	0·011	2·05	0·015	1·81	0·009

The results can be discussed from two aspects.

(*a*) *Effect of iron feeding.*

In Group II rats, which were fed a ration low in iron, the liver, spleen and body contain little more iron than do these organs from similar rats of Group I killed before commencement of feeding. Inclusion of iron in the ration of Group III has much increased the iron content, particularly in the case of liver. There is therefore some evidence to show that inorganic iron is stored in rats fed this element for a period of one month.

These remarks do not apply to the results of the third experiment, which provides no evidence of iron storage.

This disagreement regarding iron storage cannot be explained. A period of one month, however, is short and comparison of the iron content of liver from a

rat (single result) injected with 0·5 mg. iron daily for 6 months, with the content of liver from a normally fed stock rat illustrates the effect of continued dosage.

	Mg. Fe in liver	% Fe on dry matter
Rat injected	9·5	0·441
Rat normal	1·0	0·049

(*b*) *Effect of copper feeding.*

Comparison is made between Groups II and V and between Groups III and IV. In each case the only difference in the ration is the presence of copper. There is no evidence to show that copper increases the absorption or storage of iron. On the other hand, the results, while not perfectly clear cut, provide a balance of evidence to show that inclusion of copper in the diet has the effect of lowering the iron content of the liver and increasing that of the body. A possible interpretation of this is that copper promotes the utilisation of inorganic iron after it has been stored in the liver, and such an interpretation would agree with the results shown in Fig. 1, *F*.

The results just discussed together with those given in Fig. 1, *F*, showed that copper feeding probably caused utilisation of stored inorganic iron for haemoglobin building, and it was of interest to investigate this effect further. A method [Hill, 1930] became available for the determination in biological materials of "inorganic" iron as distinct from "organic" iron and some work was conducted to test the effect of copper feeding on the proportion of inorganic iron in the liver (Table XVIII). Hill has shown that $\alpha\alpha'$-dipyridyl, the reagent used in this method, does not react with reduced haematin, and the writer failed to obtain a test for iron with this reagent and the washed corpuscles of cat's blood. There are then grounds for the claim that the method will differentiate between "inorganic" and "organic" iron.

Table XVIII. *"Inorganic" and "organic" iron in livers of rats.*
(*Average values.*)

No. of rats, adults	Diet	"Inorganic" Fe		Total Fe		% Fe in "inorganic" state	% Fe in "organic" state (by difference)
		mg. Fe per liver	% on fresh matter	mg. Fe per liver	% on fresh matter		
10	Milk only	0·666	0·011	1·372	0·021	44·5	55·5
3	Milk + iron + copper	0·501	0·010	0·819	0·017	63·9	36·1
7	Milk + iron	0·650	0·011	1·403	0·023	45·2	54·8
2	Milk + manganese	0·222	0·004	0·476	0·008	46·6	53·4
2	Milk + cobalt	0·443	0·011	0·716	0·017	61·8	38·2
6	Milk + copper	0·204	0·004	0·614	0·012	28·6	71·4
3	Copper fed for 1 month Milk + copper Copper fed for 2 months	0·054	0·0008	0·361	0·005	15·2	84·8
Newly born rats	Mothers' diet						
8	Normal copper content	0·067	0·019	0·116	0·033	57·3	42·7
22	100 times normal copper content	0·034	0·013	0·077	0·030	42·0	58·0

These results indicate that the highest percentage of "organic" iron in terms of total iron was obtained in the cases where copper alone was the supplement to a milk diet: this percentage was increased by prolonged feeding

and was accompanied by a definite diminution in the total iron content of the liver. Where "inorganic" iron was added as a supplement along with the copper, there was no increase in the proportion of "organic" to total iron, probably owing to a continual addition to the stores of "inorganic" iron in the liver. Further it was observed that a high proportion of copper in the mothers' diets had a slight tendency to increase the proportion of "organic" iron in the livers of newly born rats.

No similar effects were observed when cobalt or manganese were fed as supplements without iron.

The mechanism of the action of copper in promoting haemoglobin building.

The effect of copper in promoting the utilisation of inorganic iron in haemoglobin building has been confirmed and it is of importance to consider the mechanism of this action.

The chief activity of copper is probably located in the liver, since, at all stages of growth of the animal, this organ is by far the richest in that element, and since, also, it has been shown that copper feeding causes the utilisation of iron stored in the liver for haemoglobin production (Fig. 1, *F*, and Table XVII).

Further, it has been shown, in the case of rats, that copper feeding definitely increases the proportion of "organic" to total iron in the liver, and this change from "inorganic" to "organic" iron is probably a step in haemoglobin building dependent on the presence of copper. Any modification of the state of the iron would be expected to be in the direction towards the condition of iron in haemoglobin, and it is therefore possible that copper may promote the change from inorganic iron to an iron porphyrin. Elvehjem [1931] found that the addition of copper to the nutrient medium of baker's yeast caused the formation of cytochrome a from inorganic iron, and if a similar process goes on in the rat some indication would be given that haemoglobin is derived from a haemochromogen.

It is of interest to enquire into the process whereby copper may bring about the formation of an iron porphyrin, though the suggestion to be made is, of course, entirely speculative.

(*a*) Turacin, the pigment of certain South African birds, is a naturally occurring copper porphyrin, and by chemical manipulation the copper can be displaced by iron giving a compound practically indistinguishable from haematin [Laidlaw, 1904].

(*b*) It was observed by Warburg and Krebs [1927] that after bleeding, *i.e.* when blood regeneration is rapid, the percentage of loosely bound copper in blood-serum is increased, while the percentage of loosely bound iron is decreased.

It is possible therefore, that the effect of copper in increasing the proportion of "organic" iron is achieved by a preliminary formation of a copper porphyrin and subsequent replacement of copper by iron. This would fit in with the observations outlined above and with the general chemical nature of the elements. The fact that injection of haematoporphyrin or protoporphyrin did

not increase blood regeneration (Fig. 1, *I*) indicates that the formation of the porphyrin unit itself is not the function of copper.

III. Toxicity of copper to animals.

It is now fairly generally agreed by writers on toxicology that acute poisoning by copper, resulting in death, is rare, since toxic doses are so large that they are immediately rejected by the stomach.

The position in respect to chronic copper poisoning is not so clear and whilst Pecholier and St Pierre [1856] stated that slow absorption of copper was advantageous, Mallory [1925] has expressed the view that continued exposure to copper, such as is experienced by workers in copper foundries, will cause haematochromatosis. In support of Mallory, Funk and St Clair [1930] have reported a case of haematochromatosis in man where the proportion of copper in the liver was extremely high.

Feeding experiments with animals have been conducted chiefly with rabbits, the procedure being to feed copper acetate for a period and to observe the extent of pigmentation in the livers. No detailed observations regarding growth, fecundity *etc.* of animals receiving an abnormally high ration of copper have yet appeared, and experiments on this aspect have therefore been conducted with rats.

The experiments included, in all, six groups—the same basal ration being used throughout but with differing amounts of copper salts included in the salt mixture for Groups II–VI.

Details of the groups were as follows:

Group I. 14 newly weaned rats, 9 females and 5 males. Basal ration 0·011 mg. copper per rat daily.

Group II. 14 newly weaned rats, 9 females and 5 males. Copper sulphate added to give 0·050 mg. copper per rat daily.

Group III. 8 newly weaned rats, 5 females and 3 males. Copper sulphate added to give 7·5 mg. copper per rat daily.

Group IV. 5 adults, 3 females, 2 males. Copper sulphate added to give 7·5 mg. copper per rat daily.

Group V. 5 adults, 3 females, 2 males. Copper acetate added to give 7·5 mg. copper per rat daily.

Group VI. 5 adults, 3 females, 2 males. Basic copper acetate (verdigris) added to give 7·5 mg. copper per rat daily.

The basal ration low in copper was as follows:

Kraft cheese	45 g.
Butter	7·5 g.
Maize starch	65 g.
Castor sugar	35 g.
Salt mixture I	5 g.
Radiostol	3 drops
Milk	150 cc.

This weight of food was sufficient for fifteen rats.

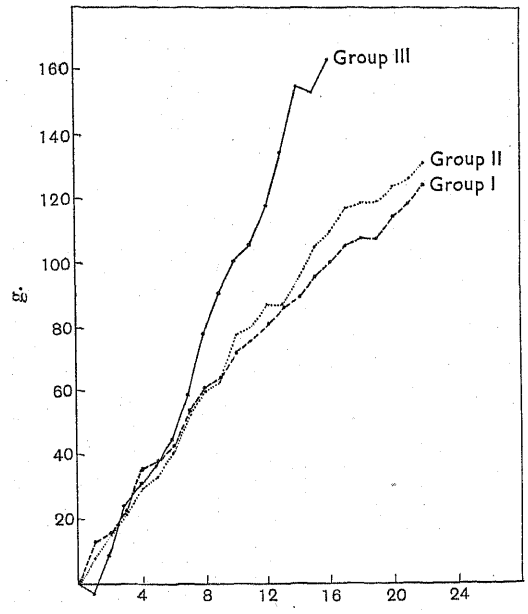


Fig. 4. Average gain in weight of rats.

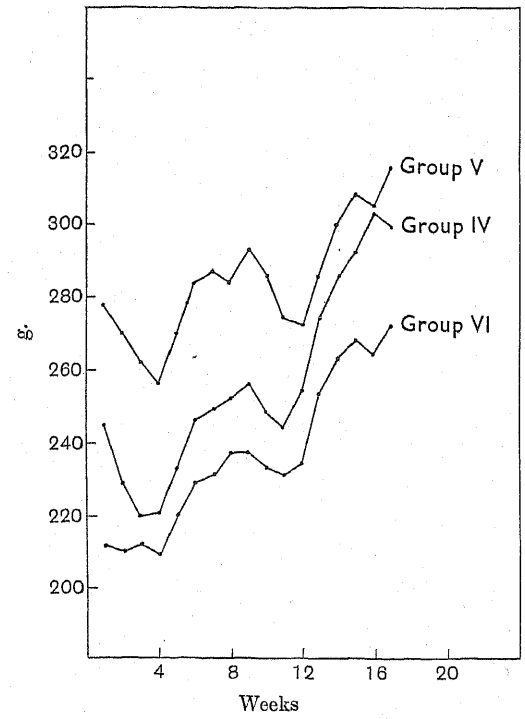


Fig. 5. Average weight of rats.

The rats were weighed weekly. For Groups I–III the average gains for one rat at weekly intervals are plotted in Fig. 4, although the growth curve for Group III is not strictly comparable with curves for Groups I and II since the former group was started at a different time of the year. In Fig. 5 are plotted the average weights per rat for each of groups IV–VI.

Haemoglobin determinations were made at irregular intervals on all rats and normal values were found without exception.

Table XIX contains the reproduction records of rats from each group. Some of the rat pups were killed at birth and analysed for copper and iron; the results are also included. The remaining young were reared successfully in all cases.

Table XIX.

Group	I	II	III	IV	V	VI	Stock diet; 1 litter for comparison.
Copper in diet per rat daily:	0·011 mg. $CuSO_4$	0·050 mg. $CuSO_4$	7·5 mg. $CuSO_4$	7·5 mg. $CuSO_4$	7·5 mg. Cu acetate	7·5 mg. verdigris	About 0·05 mg. Cu
No. of females in group	8	8	5	3	3	3	—
No. of litters	8	9	4	3	3	Nil	—
No. of pups per litter (av.)	6·9	7·9	6·3	8·0	6·7	—	8
Av. wt. of one pup at birth (g.)	4·8	6·6	6·0	5·7	4·8	—	5·0
Total number analysed	24	21	Nil	13	7	—	8
Mg. Fe/rat pup (av.)	0·268	0·312	—	0·352	0·316	—	0·275
Mg. Cu/rat pup (av.)	0·0045	0·0073	—	0·0145	0·0147	—	0·0046
% Fe on dry matter (av.)	0·0374	0·0320	—	0·0412	0·0467	—	0·0354
Mg. Cu/kg. dry matter (av.)	6·3	8·0	—	16·2	20·9	—	5·9

The following conclusions may be drawn from this experiment.

(1) The first generation of rats throve on a diet providing as little as 0·011 mg. copper to each rat daily. In comparison with rats receiving a normal amount of 0·05 mg. copper daily the low-copper rats grew at a slightly slower rate, the number of pups produced in one litter was slightly less and the average weight of individual rat pups was somewhat smaller. Differences were not marked and sufficient data are not available to decide whether these are significant. At the same time, since the ration proved to be a good one, the low-copper rats grew rather faster than is normally the case for the stock colony, and their haemoglobin was maintained at a normal level.

(2) Analyses of the young produced by these two groups of rats showed on the average no marked difference in iron content but the copper content was significantly lower in those pups whose mothers were fed a low ration of copper. It will be seen that there is a further increase in the copper content of young rats from breeding mothers receiving 7·5 mg. copper per day. There are then,

some indications that the copper content of the diet of rats will affect the storage of copper in the foetus. Hart *et al.* [1930] have shown that the copper content of the diet of sows has no effect on the copper transmitted to the young pigs. These differences between the two species perhaps have some bearing on the facts that while copper promotes the utilisation of inorganic iron for haemoglobin building in the rat it has been claimed that in the pig [Hart *et al.*, 1930] it is not necessary for this purpose.

(3) The presence in the diet of over 100 times the normal amount of copper (as copper sulphate) has no detrimental effect on the growth of rats. Normal young may also be produced on such a ration.

(4) The presence in the diet of over 100 times the normal amount of copper as copper acetate or as verdigris was not toxic to adult rats. Again, histological examination of livers after 5 months' feeding did not reveal any deposition of pigment. Breeding was normal in those rats fed copper acetate but no litters were produced in the same time by the animals receiving verdigris.

Summary.

1. Determinations of copper have been made in plants, foodstuffs, insects and marine animals. A systematic survey has also been made of the occurrence of copper in the organs of twelve species of land animals (foetal, newly born and adult).

2. The copper content of the skin and of the livers of rats has been shown to be increased in relatively small degree compared with the additional copper fed in the diet. Feeding additional copper to the mother caused increased storage of copper in the newly born rat.

3. A catalytic effect of copper on the oxidation of "dopa" to melanin has been observed. A suggestion has also been made that the copper in the ink sac of the octopus plays some part in the formation of the ink.

4. Copper feeding has been shown to have little or no effect on the storage of iron in the foetal rat or in the hen's egg.

5. Iron feeding has been shown to have no effect on the storage or excretion of copper by the rat.

6. The use of milk diets for production of a nutritional anaemia in rats has been criticised. At the same time the stimulating effect of copper on the formation of haemoglobin in rats has been confirmed and a possible mechanism suggested for its action.

7. It has been shown that feeding high amounts of copper sulphate or copper acetate has no effect on the growth and reproduction of rats, while high amounts of verdigris were observed to stop reproduction. It has also been shown that rats receiving one-fifth of the normal amount of copper in the diet will grow and reproduce normally.

The work reported above was carried out during a visit of the writer from New Zealand. This visit put into effect a scheme for the interchange of workers

in Agricultural Science, proposed at the Imperial Agricultural Conference in 1927. I desire to express my appreciation of the opportunity thus afforded, of carrying out research work in this country.

I also wish to express my very great indebtedness to Mr W. Godden for his continued interest and for his counsel throughout the investigation, and to Dr J. P. McGowan for his advice on problems of pathology and haematology.

REFERENCES.

Aronssohn (1911). *Compt. Rend. Acad. Sci.* **152**, 1183.
Aston (1911). *Trans. New Zealand Inst.* **44**, 288.
—— (1921). *New Zealand J. Agric.* **22**, 122.
Beard and Myers (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 510.
Biazzo (1926). *Ann. Chim. Appl.* **16**, 2.
Bloch (1927). Handbuch für Haut- und Geschlechtskrankheiten.
Boycott (1911). *J. Path. Bact.* **15**, 388.
Bunge (1902). Physiol. und pathol. Chem. (Eng. ed.).
Church (1892). *Proc. Roy. Soc. Lond.* **51**, 399.
Drabkin and Miller (1931). *J. Biol. Chem.* **90**, 531.
—— and Waggoner (1930). *J. Biol. Chem.* **89**, 51.
Elvehjem (1931). *J. Biol. Chem.* **90**, 111.
—— and Hart (1929). *J. Biol. Chem.* **82**, 473.
Fleurent and Levi (1920). *Bull. Soc. Chim.* **27**, 441.
Fox and Ramage (1931). *Proc. Roy. Soc. Lond.* B **108**, 157.
Funk and St Clair (1930). *Arch. int. Med.* **45**, 37.
Giunti (1879). *Gazzetta*, **9**, 546.
Guérithault (1920). *Compt. Rend. Acad. Sci.* **171**, 196.
Haber (1926). *Bull. Brooklyn Ent. Soc.* **21**, 61.
Hart, Elvehjem, Steenbock and Kemmerer (1930). *J. Nutrition*, **2**, 277.
—— Steenbock, Waddell and Elvehjem (1928). *J. Biol. Chem.* **77**, 797.
Hill (1930). *Proc. Roy. Soc. Lond.* B **107**, 205.
Javillier and Guérithault (1927). *Compt. Rend. Acad. Agric.* **13**, 96.
Keil and Nelson (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 392.
Koller (1930). *J. Genetics*, **22**, 103.
Krauss (1930). *J. Dairy Sci.* **13**, 246.
—— (1931). *J. Biol. Chem.* **90**, 267.
Krumbhaar (1930). *Sang*, **4**, 267.
Laidlaw (1904). *J. Physiol.* **31**, 464.
Lindow, Elvehjem and Peterson (1929). *J. Biol. Chem.* **82**, 465.
—— Peterson and Steenbock (1929). *J. Biol. Chem.* **84**, 419.
Mallory (1925). *Amer. J. Path.* **1**, 117.
Maquenne and Demoussy (1920). *Bull. Soc. Chim.* **27**, 266.
Mayer, Borchardt and Kikuth (1927). Suppl. to *Arch. Schiff. Tropen-Hyg.* **31**.
McCollum and Becker (1930). *Amer. J. Hyg.* **12**, 503.
McGowan (1924). *J. Path. Bact.* **27**.
McHargue (1925, 1). *J. Agric. Res.* **30**, 193.
—— (1925, 2). *Amer. J. Physiol.* **72**, 583.
—— (1926). *J. Amer. Soc. Agronomy*, **18**, 1076.
—— Healy and Hill (1928). *J. Biol. Chem.* **78**, 637.
Meissner (1816). *Schweigger's J.* **17**, 350.
Meyer (1929). *Zentr. Bakt. Par.* I, **110**, 152.
Mitchell and Miller (1929). *J. Biol. Chem.* **85**, 355.
Morrison and Nash (1930). *J. Biol. Chem.* **88**, 479.
Muttkowski (1921). *Ann. Ent. Soc. Amer.* **14**, 150.

Pecholier and St Pierre (1856). Chimie appliquée à la physiologie et à la thérapeutique.
Powick and Hoagland (1924). *J. Agric. Res.* **28**, 339.
Quartaroli (1928). *Ann. Chim. Appl.* **18**, 427.
Rose and Bodansky (1920). *J. Biol. Chem.* **44**, 99.
Severy (1922). *J. Biol. Chem.* **55**, 79.
Stewart and Percival (1930). *Brit. J. Dermat. Syphilis*, **42**, 215.
Titus and Hughes (1929). *J. Biol. Chem.* **83**, 463.
Waddell, Steenbock, Elvehjem and Hart (1928). *J. Biol. Chem.* **77**, 769.
—— Steenbock and Hart (1929). *J. Biol. Chem.* **84**, 115.
Warburg and Krebs (1927). *Biochem. Z.* **190**, 143.
Webster and Jansma (1929). *Science*, **70**, 174.
Weichselbaum (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 329.
Wells and Mehta (1930). *Brit. Med. J.* i, 1167; and *Indian J. Med. Res.* **17**, 777.

CXLII. ON THE FUNDAMENTAL NATURE OF VITAMIN D ACTION.

BY JOHN POOL McGOWAN, IRA JAMES CUNNINGHAM AND DOUGLAS WILLIAM AUCHINACHIE.

(Preliminary Communication.)

From the Rowett Research Institute, Aberdeen.

(*Received July 4th, 1931.*)

IN a previous paper [McGowan, 1930] it was shown that, in chloroform poisoning in the rabbit, there is an accumulation of fatty substances together with calcium in certain of the tissues, especially the liver. This was interpreted as meaning that chloroform attacked and broke up the lipins, thereby setting phosphoric acid free to be neutralised *in situ* by calcium to form insoluble calcium phosphate. A similar deposition of calcium salts in certain of the tissues, following on a preliminary degeneration, has been recorded as the main feature of vitamin D poisoning [Duguid, 1930; and others]. Experiments were therefore undertaken to see if this hypervitaminosis D was explicable on a similar basis.

Rabbits were employed. These, for several weeks prior to the experiment, had been kept on the experimental ration, which was a modification of the stock ration in the direction of a considerable increase of its calcium carbonate content. The stock ration consisted of bran 8 parts, white sharps 8, bruised oats 8, and fish meal 1. To this was added, to form the experimental ration, 2 parts calcium carbonate, making thus roughly 8 %.

The rations were fed *ad lib.* and, in every case, in addition there was an abundant supply of freshly cut clover-rich grass. Water, for drinking purposes, was always available.

The vitamin D employed was in the form of a solution of irradiated ergosterol in olive oil, of a strength of 1,000,000 rat units per cc. For a supply of this we are greatly indebted to Mr A. L. Bacharach of the Glaxo Research Laboratories. It was administered by mouth by means of a pipette twice daily. So far the dosage employed has been 4–8 million rat units per day.

In Table I are summarised some results obtained in illustrative cases.

Table I deals with results obtained from a rabbit (No. 2) poisoned with vitamin D; a rabbit (No. 4) poisoned with chloroform; normal rabbits (Nos. 5, 10 and 11), fed on the stock ration for the composition of normal urine for comparison purposes; and two normal rabbits for the calcium content of the liver.

Table I.

Rabbit and dose	Diet	Date	Weight g. A	Cc. urine in 24 hrs. B	Nature of deposit, *etc.* Crystals C	Gas with acid D	Urine reaction C	Total CaO as carbonate % E	Total CaO % F	Total P_2O_5 % G	CaO in 24 hrs. g. H	P_2O_5 in 24 hrs. g. I	Ca in blood mg./100 cc. J	P in blood mg./100 cc. K	Ca % moist weight liver L	Ca % moist weight kidney M
No. 2. Irradiated ergosterol, 4 million rat units daily commencing 11. vi. 31	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	10. vi. 31	2020	72	Almost pure $CaCO_3$	+ + + + + +	Alk.	—	0·36	0·01	0·259	0·007	17·5	3·03	—	—
		11. vi. 31	2030	150	do.	+ + + + + +	do.	—	0·44	0·01	0·660	0·015	—	—	—	—
		12. vi. 31	1950	121	Almost pure amorphous phosphates	+	do.	—	0·64	0·13	0·774	0·157	—	—	—	—
		13. vi. 31	1950	65	do.	+	do.	—	0·57	0·46	0·370	0·299	—	—	—	—
		14. vi. 31	1840	72	do.	+	do.	0·13	0·42	0·47	0·302	0·338	—	—	—	—
		15. vi. 31	1795	85	do.	+	do.	0·04	0·50	0·46	0·425	0·391	—	—	—	—
		16. vi. 31	1750	*												
		16. vi. 31	Died	22·5†	do.	+	do.	0·17	0·64	0·29	—	—	—	—	0·0076	0·23
No. 4. 1·0 cc. $CHCl_3$ on 17. vi. 31 subcutaneously	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	15. vi. 31	1590	—	—	—	—	—	—	—	—	—	—	—	—	—
		16. vi. 31	1650	274	Almost pure $CaCO_3$	+ + + + + +	Alk.	0·60	0·61	0·02	1·671	0·055	15·3	5·3	—	—
		17. vi. 31	1640	145	do.	+ + + + + +	do.	0·95	1·17	0·03	1·697	0·044	—	—	—	—
		18. vi. 31	1600	82	Almost pure amorphous phosphates	+ ?	Neutral	0·00	0·33	0·43	0·271	0·353	13·8	3·2	—	—
		19. vi. 31	1550	144	do.	+	Alk.	0·03	0·15	0·08	0·216	0·115	—	—	—	—
		19. vi. 31	Killed	25†	do.	+	do.	0·05	0·14	0·05	—	—	—	—	0·0078	0·028
Normal. No. 5	Stock ration alone + grass	20. vi. 31	1800	130	None	None	Acid	None	0·04	0·26	0·052	0·338	14·8	3·2	—	—
No. 10		20. vi. 31	2350	115	do.	do.	do.	do.	0·009	0·46	0·010	0·520	—	—	—	—
No. 11		20. vi. 31	1900	50	do.	do.	do.	do.	0·039	0·18	0·078	0·360	—	—	—	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0065	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0066	—

* Urine lost by being passed into food dish. † In bladder.

Column A shows the loss of weight from vitamin D and chloroform poisoning. Rabbit 2 ceased to eat on the 14th, two days after vitamin D administration commenced, whereas rabbit 4 stopped on the 17th, as soon as the chloroform had been administered. These occurrences have to be considered in relation to the results obtained below.

Column B gives the amount of urine passed in 24 hours. When the animal died or was killed, the urine present in the bladder was also recovered.

Column C deals with the reaction and the nature of the deposit present in the urine as observed microscopically. In the rabbits on high-calcium diet, the urine was alkaline; on stock ration, acid. In rabbits 2 and 4 on high-calcium diet, the deposit prior to poisoning consisted practically entirely of calcium carbonate crystals. Immediately on poisoning, the calcium carbonate was replaced by amorphous phosphates. There was no deposit present in the normal rabbits on stock ration.

In Column D is given the effect of adding acid to the microscopical preparations. For comparative purposes, a record in arbitrary symbols is given of the amount of gas (CO_2) evolved. It will be seen that this supports the findings in Column C.

In Column E is recorded, as CaO % in the urine, the amount of calcium present as carbonate, as calculated from the amount of gas collected on making the urine distinctly acid. This line of observation was not adopted till 14. vi. 31, hence the results for rabbit 2 prior to this date are lacking. From those obtained in Column D, however, they are undoubtedly quantitatively much the same as in rabbit 4 for the pre-poisoning period. The great diminution of the calcium in the form of carbonate in the poisoning period is again evident, while the very great excess of calcium as carbonate or phosphate in the urine of the experimental animals over that in the urine of the normal rabbits on the stock ration is demonstrated.

Columns F and G give respectively the percentage total CaO and P_2O_5 in the urines. These results, however, can only be discussed in relation to Columns H and I.

In regard to Column H, it will be seen that in rabbit 2 the calcium in the poisoning period remains more or less at the level it was in the pre-poisoning period, with the exception of a possible fall at the very end (see Column F), whilst in rabbit 4 there is a very marked drop in the poisoning period. The result in rabbit 4 may be connected with the diminished intake of food, with its high calcium content, which occurred during this period.

In Column I is demonstrated the very marked increase in the poisoning period of the P_2O_5, more gradual in the case of rabbit 2, with a definite falling off in rabbit 4 and a possible one also in rabbit 2 towards the end of the period. This terminal fall may possibly be attributed to metabolism as a whole having more or less ceased, owing to the animal being *in extremis*, or to the supply of readily available lipins, from which phosphorus could be split off, being used up. The results, therefore, as far as regards the phosphorus, indicate a greatly

increased excretion of phosphorus, which agrees with Duguid's summing up of the situation [1930], where he states that "there is a very greatly increased excretion of phosphorus especially by way of the kidneys and this, occurring when the food consumption is diminishing as is usual in hypervitaminosis D, involves a reduction in the phosphorus retention in the body." Duguid [1930] also states that an increased excretion of calcium in the urine is the rule in such cases. The different result obtained in the present instance may have reference to the very high calcium content of the food and the high level of calcium excretion in the urine to begin with.

The results in Columns E, H and I go to show that the calcium, formerly excreted as carbonate, is got rid of in the poisoning period in the form of phosphate.

The bones of the rabbits were ivory hard and showed no trace of thinning of the cortical bone or of any demineralisation process.

In Columns J and K a few results are given of the estimation of Ca and P in the circulating blood. This mode of observation, however, was not pressed. It was recognised that the withdrawal of 10 cc. of blood from a rabbit at frequent intervals would jeopardise more important results, while it was also borne in mind that, in the poisoning stage, owing to the depressed condition of the circulation, it is often impossible to obtain a sample of blood. More important still, it was realised that, in such procedures, one is estimating the Ca and P in a sample of blood taken in its passage from an irregular and varying "source" or "sources" to an irregular and varying "distribution," two variables regarding the essential activity of either of which, at the time the blood sample was being taken, there is and can be, under present circumstances, no real knowledge. Thus, while a high Ca % in the blood might mean a high intake from the "sources," it might equally well denote a diminished "distribution." A similar state of matters exists in regard to phosphorus. There is also possible a variety of further permutations and combinations of "source" and "distribution" with an effect on the blood composition.

In a previous communication [McGowan, 1930, Table, p. 288], it was shown that in chloroform poisoning there was a great increase in the amount of fat and calcium salts in the central two-thirds of the liver lobules. The amounts present of these two substances ran more or less parallel. Where relatively small amounts of each existed, the histological picture showed that these were remnants, left from large amounts previously present, which, in the meantime, had been removed by the circulation. This process of removal is a rapid one, for the experiments lasted in most cases for a few days only. In these experiments also, the fatty material was present not only as neutral fat but to a large extent as calcium soap. For comparison with the figures obtained in the present investigation, it may be stated that, in one of these cases of chloroform poisoning in the rabbit, the calcium as metal present in the liver was as much as 2·66 % of the moist weight. On the other hand, figures as low as 0·025 % were obtained.

In the present case of chloroform poisoning (rabbit 4) the findings were quite different from those just stated. The inner two-thirds of each lobule was indeed crammed with lipoid material, which, on staining with Nile blue sulphate, was found to be neutral fat without any fatty acids. Von Kossa's silver nitrate test for calcium phosphate gave entirely negative results, while the amount of calcium present was only 0·0078 % of the moist weight, a figure differing but little from the normal (*vide* bottom of column L). It is difficult to explain the anomalous condition present here unless one assumes that the large amount of $CaCO_3$ in the diet had prevented the absorption of inorganic phosphates from the food, thus rendering the blood relatively deficient in, and avid for, inorganic phosphate and so withdrawing the latter from the tissues whenever and as soon as it was formed [*cf.* Cushny, 1918].

If one now turns to consider the appearances present in the livers of the rabbits poisoned with vitamin D, frozen sections stained with Sudan III gave no evidence of the presence of fatty material in any considerable amount. In this connection, however, it should be noted that Haendel and Malet [1930] have found large quantities of fat in the liver, kidney, heart and spleen of rabbits poisoned with vitamin D. Von Kossa's stain also gave a negative result with regard to the presence of calcium phosphate. Fat, nevertheless, may have been present in the cells of the present cases in globules too minute to be detected by the coarse technique just alluded to. Osmic acid preparations have not as yet been made, but ordinary paraffin sections show a very advanced stage of cloudy swelling, with granular degeneration and a basophil reaction of the cytoplasm, conditions found present also in the liver cells at the periphery of the liver lobules in chloroform poisoning. This latter appearance may indicate a reaction to the acid side, which may possibly be due to acid phosphates, derived from a breaking down of the lipoids. In any case, cloudy swelling is the well-known precursor of the ordinary easily recognisable fatty degeneration. The calcium present varied little from normal, being 0·0076 % of the moist weight.

The histological changes in the various organs and tissues of the rabbits poisoned with vitamin D may now be discussed. They were practically those found by previous observers. The following tissues were examined: liver, spleen, kidney, testicle, suprarenal, heart muscle, wall of aorta, lung, skeletal muscle, stomach wall, small intestine, colon and bone marrow. Briefly put, the changes observed were, in the first place, cloudy swelling and granular degeneration, especially present in the liver cells, secretory tubule cells of the kidney and in the muscle of the heart, arteries and stomach wall. Deposition of calcium salts was found present in the media of the arterioles of the cortex of the kidney and of the lung and in the media of the aorta. Specially, there was no deposition in the heart muscle or in the arterioles of the heart. There were large patches of precipitation in the muscular wall of the stomach of such size that they could be seen by the naked eye as white striations. A few small spots were also present in the gastric mucosa. As already suggested in relation

to rabbit 4, the high calcium diet may have interfered somewhat with calcium deposition. The kidney showed the presence of calcium in its tubules. Doubtless this is to be explained by the high excretion of calcium in the urine and is not necessarily indicative of a degenerative change in the epithelium. The relatively high calcium in the kidney (*vide* Column M) seems also explicable on this basis, the higher figure for rabbit 2 being an index of the increased calcium in the walls of the arterioles.

The suprarenals in these cases were much enlarged, the diameter being about five times that of the normal gland. The enlargement was mainly in the cortex at the expense of the medulla. The cortex was pure white in colour and gave a marked Sudan III reaction, while in paraffin sections the cytoplasm of the cells stained deeply basophil. The significance of these findings awaits elucidation.

Contrasting the condition found in these cases with that present in chloroform poisoning, it would seem that, in both instances, there is an attack on the protoplasm of the cell. In chloroform poisoning, however, there is a much larger amount of the poison active at any one time, the condition being, in every respect, a more fulminating one. The poisoning produces its maximum effect almost at once, to be followed almost immediately by phenomena of recovery. Hence the lipins are broken up rapidly, fatty bodies and calcium phosphate being produced in large abundance *in situ* without time being given for their removal into the circulation. Thus, within the time employed for such investigations, in most instances they remain to some degree in the tissues and are observed on histological examination. Evidence has, however, already been adduced to show that they can be, and are at times, fairly rapidly removed [McGowan, 1930, Table, p. 288].

In the case of vitamin D poisoning, however, the amount of poison active at any one time is very small, the attack on the cell and its lipins is not so drastic, and ample time is given for the removal of by-products. Hence, on histological examination, the appearances found are much milder in type and calcium is found only in those situations, the media of the arterioles, *etc.*, where, once having been deposited, it cannot so readily be removed as it can in the case of parenchymatous cells of actively metabolising glands, like the kidney and liver, which abut on a rich blood supply. This view is supported by the results obtained by Hertzenberger [1929], who found evidence of the healing of the lesions and disappearance of the calcium deposits after cessation of administration of vitamin D and by those of Schiff [1930], who found no evidence of resorption of the calcium from the arterial lesions after poisoning was stopped.

The rather insignificant pathological findings just described have been obtained in the case of an animal which actually died within 5 days from the effects of the poisoning. It is a question therefore whether changes of this degree are not the essential ones and whether the more advanced changes, involving parenchymatous cells of various organs in fatty change and calcium

deposition, which have been described, may not have to be attributed to an anoxaemia brought about by the alterations in the vascular walls.

The broad aspects of the subject in hand may be discussed and the position summed up by a consideration of some points raised by Cushny [1918] relative to phosphorus and calcium metabolism. On p. 108, he makes the statement that in the herbivora phosphates are excreted exclusively by the bowel wall. Obviously, this generalisation is incorrect, for, in Table I, it will be seen that rabbits 5, 10 and 11, on the stock ration, pass considerable quantities of phosphates in the urine. Dealing with the subject of calcium metabolism at p. 560, Cushny states that calcium is excreted in part by the urine but for the most part through the epithelium of the large intestine. The relative amounts excreted by the kidney and bowel seem to be determined by the quantity of available phosphates among other factors. If these are present in large quantities in the blood, the calcium is excreted mainly in the bowel in the form of calcium phosphate. The elimination of calcium thus appears to vary with the character of the combinations which it can form. If these are soluble they appear in the urine, while the insoluble ones tend to pass into the stools. Calcium lessens the phosphates of the urine by forming insoluble phosphates in the bowel and thus preventing the absorption of the phosphates of the food.

It seems possible to harmonise these views of Cushny with the results obtained in our experiments. It may be supposed that, in the rabbits fed on the stock ration, there is not enough calcium to join up with the phosphate and carry it in the insoluble form of calcium phosphate to the bowel for elimination. There is an over-plus of phosphate which appears in the urine as alkaline phosphates. With more calcium in the food, it could be supposed that practically all the phosphate goes as calcium phosphate to the bowel, leaving only a trace to appear in the urine. *A fortiori*, this holds for conditions where there is great excess of calcium in the diet, with the further significant occurrence that the excess calcium appears in the urine as the carbonate conveying CO_2 out of the body, a function more efficiently performed by the lungs. In conditions of vitamin D and chloroform poisoning in animals fed excess of calcium carbonate, there is great excess of phosphate produced, more than can be got rid of as calcium phosphate by the intestine. The excess calcium phosphate produced now goes by the kidney, the calcium formerly excreted as carbonate being utilised for this purpose. One slight blemish—it is, however, a slight and unimportant one from the present point of view—appears at first sight to mar the complete correlation. Cushny states that the insoluble calcium compounds pass by the bowel and apparently by the bowel only. Yet, it is indubitable that, in the present experiments, not only has calcium phosphate passed by the kidney (for this of course an explanation has been offered), but also such an insoluble substance as calcium carbonate. Doubtless, however, Cushny's statement will hold if the food supplies no more calcium than can be dealt with by the phosphate in the blood and so be removed as insoluble calcium phosphate by the intestine.

It seems possible that the findings of Harris and Innes [1931] that vitamin D poisoning is accentuated by increase of calcium in the diet, but that vitamin D, even in large doses over a long period, becomes relatively harmless when calcium is balanced by the addition of phosphate or when calcium is omitted and phosphate remains high, may derive some explanation from what has just been discussed. For one thing, there will be less calcium present to form deposits and, for another, the less speedy "insoluble" method of elimination by the bowel in the form of calcium phosphate may be supplanted by the more speedy "soluble" method of elimination by means of the kidney in the form of acid phosphates of the alkali metals.

The subject remains for discussion as to the relation of the present findings to the question of the nature of rickets and of the action of vitamin D in this disease. Elsewhere [McGowan, 1926], it has been pointed out (and the implications emphasised) that rickets begins very early during the suckling stage of the animal. Evidence was also adduced to show that the appearance of rickets depends on a deficiency of consolidating materials in the milk relative to the growth-promoting ones and to the growth potential of the young animal. Further, it has been shown that the administration of inorganic phosphate, in the form of sodium phosphate, can prevent and cure rickets; in the fowl, by Bethke, Kennard, Kick and Zinzalian [1927–28] and in the rat by Karelitz and Shohl [1927] and Kramer, Shear and Siegel [1931]. From our previous discussion and these considerations, we draw the conclusion that there is more to be said for a relative deficiency of inorganic phosphate being the actual *causa causans* of rickets than for the lack of any other substance hitherto alleged to be operative[1].

As to the possible mode of action of vitamin D in combating the disease, apart from what has already been adumbrated above in this direction, an insight may be gained by a consideration of the significance of the persistent low blood phosphorus that occurs in rickets. This low phosphorus may be attributed, on the one hand, to the constant removal, for constructional purposes, from the blood of inorganic phosphorus by the avid bony tissues and, on the other hand, to a low supply to the blood from the diet which is deficient in inorganic phosphorus. This can be rectified, directly, by supplying additional inorganic phosphorus in the diet or, indirectly, by supplying a substance—vitamin D—which will make available to the blood the inorganic phosphorus stored in the body lipins. Further, it seems obvious that the supply of great excess of inorganic calcium in the diet, by its action in rendering the inorganic phosphates of the diet insoluble and unabsorbable, will predispose to the production of rickets.

[1] A calcium-deficient ration produces osteoporosis, not rickets. At the same time, it should be noted that rickets is not, in our opinion, due essentially and primarily to a lack of vitamin D.

SUMMARY.

Evidence has been adduced to show that the probable mode of action of vitamin D in the cure and prevention of rickets is by the setting free from the lipins of the body of inorganic phosphate, the relative deficiency of which is regarded as the essential cause of rickets.

REFERENCES

Bethke, Kennard, Kick and Zinzalian (1927–28). *Poultry Sci.* **8**, 259.
Cushny (1918). Pharmacology and therapeutics, 7th edit. (Churchill, London.)
Duguid (1930). *Lancet*, ii, 983.
Haendel and Malet (1930). *Virchow's Arch.* **276**, 1.
Harris and Innes (1931). *Biochem. J.* **25**, 367.
Hertzenberger (1929). *Beitr. path. Anat. allgem. Path.* **82**, 27.
Karelitz and Shohl (1927). *J. Biol. Chem.* **73**, 665.
Kramer, Shear and Siegel (1931). *J. Biol. Chem.* **91**, 271.
McGowan (1926). Pernicious anaemia, leukaemia and aplastic anaemia, Chap. IX. (H. K. Lewis and Co., London.)
—— (1930). *Edin. Med. J.* **37**, 281.
Schiff (1930). *Virchow's Arch.* **278**, 62.

CXLIII. STUDIES ON HEMICELLULOSES.
IV. THE PROXIMATE ANALYSIS OF BOX-WOOD, AND THE NATURE OF ITS FURFURALDEHYDE-YIELDING CONSTITUENTS.

By ISAAC ARTHUR PREECE.

From the Department of Biochemistry, University of Birmingham.

(*Received June 27th, 1931.*)

Introduction.

As a preliminary to an investigation of the nature of the hemicelluloses of box-wood, it was desired to obtain as complete an idea as possible of the constitution of the wood, and with the minimum of overlapping among the constituents estimated.

Preliminary experiments had indicated that the furfuraldehyde-yielding constituents of the wood could be separated into a number of groups. Thus, hot 0·5 % ammonium oxalate solution removed from the sawdust both pectin and a small amount of non-pectic substance capable of yielding furfuraldehyde. Again, extraction of the residue from this treatment with boiling alcoholic sodium hydroxide solution dissolved in addition to lignin a substance which was reprecipitated on rendering the extract acid. This product, here described as "lignosaccharide," appeared to resemble the hemicelluloses closely in properties and constitution. Extraction with 4 % sodium hydroxide solution at room temperature removed "free hemicelluloses," but a portion of the hemicellulosic substance still remaining in association with the tissue residue required more drastic treatment for its removal. This "combined hemicellulose" was dissolved out by hot 4 % sodium hydroxide solution, but an amount of "other pentosans" remained undissolved, and this was shown to consist largely, if not entirely, of xylan.

Means were found for estimating separately all the above groups with the exception of the non-pectic substance soluble in ammonium oxalate solution. This substance was also found to be soluble in hot water and is included in the results of analysis under the heading "hot water-soluble." In addition, a number of other determinations were made, including ash, alcohol-ether-soluble substances, protein, lignin and cellulose, and it is thought that no constituent of the wood present in significant amount escapes inclusion under these headings.

EXPERIMENTAL.

A. Proximate analysis.

Methods of analysis.

Furfuraldehyde-yielding constituents. The estimation of hemicelluloses is a matter of some difficulty. The alkali-soluble "furfuroids" of Cross, Bevan and Smith [1895] would correspond roughly to hemicelluloses, but the figures obtained by these authors can only be approximations. Konig and Becker [1919] determined hemicelluloses in wood by heating with 0·4 % sulphuric acid at arbitrary pressures, the sugars arising being estimated in the resultant hydrolysis liquid. Dore [1920] determined the loss in weight of woods on extraction with 5 % NaOH. Other workers have distinguished "pentosans in cellulose" and "pentosans not in cellulose."

A method for the determination of hemicelluloses in cereal straws has recently been described by Norman [1929], it being assumed that the only furfuraldehyde-yielding constituents of the straws are pectin, free hemicelluloses and xylan associated with cellulose. Attempting to apply this method of estimation to box-wood, the following figures were obtained.

Table I.

Total furfuraldehyde on dry wood	11·86 %
Calcium pectate yield	0·41
Cellulose (Cross and Bevan)	49·35
Furfuraldehyde yield % of cellulose (Cross and Bevan)	10·35
% xylan in cellulose	16·04
% xylan calculated to dry wood	7·92

It may be pointed out that a knowledge of the carbon dioxide yield of wood and hemicellulose, obtained by the method of Nanji, Paton and Ling [1925], is not necessary to the calculation.

100 parts calcium pectate yield 19·5 parts furfuraldehyde.
100 parts xylan yield 64·5 parts furfuraldehyde.

Hence, furfuraldehyde arising from hemicellulose is given by

$$11{\cdot}86 - \{(0{\cdot}41 \times 0{\cdot}195) + (7{\cdot}92 \times 0{\cdot}645)\} = 6{\cdot}67\ \%.$$

The yield of furfuraldehyde from the hemicellulose being 55·14 %, the percentage of hemicellulose on the dry wood should be given by the quotient of 6·67 and 0·5514, *i.e.* 12·09 %. However, carefully carried out preparations of the hemicellulose mixture gave yields amounting to no more than two-thirds of this value, and similar discrepancies were found with other woods. It is therefore apparent that this method of determination is not applicable to woods.

It was decided, therefore, in the present work to determine the hemicelluloses by actual preparation, by a method which is in essentials that described by Norris and Preece [1930].

20–25 g. of the wood are extracted on the boiling water-bath with 300 cc. of 0·5 % ammonium oxalate solution for 1½ hours, the mass after cooling being filtered on paper at the pump. The residue is washed back into the extraction flask and extraction with ammonium oxalate is repeated. The filtered residue is then treated for 1½ hours with 300 cc. of boiling 50 % alcohol containing 1 % of sodium hydroxide. After further filtration through paper at the pump, the residue is submitted to two further extractions with 300 cc. portions of 50 % alcohol. The three filtered alcoholic extracts are combined and reserved.

The filtered residue, thus freed from pectin and much of its lignin, is allowed to stand at room temperature with 300 cc. of 4 % NaOH solution for not less than 6 hours. The extract is then filtered at the pump through a circle of calico previously boiled with 4 % NaOH. The wood residue is repeatedly further extracted with 150 cc. portions of NaOH solution until the filtered extract no longer gives a precipitate on neutralising with glacial acetic acid and adding an equal volume of acetone.

The combined filtrates are rendered acid by addition of glacial acetic acid, and a volume of acetone equal to that of the acid liquid is added. The precipitate is allowed to settle, the supernatant liquor syphoned off and the hemicellulose treated with graded strengths of alcohol, drying being continued *in vacuo* after filtering at the pump. The weight of the product is ascertained, its moisture content determined on a small portion by oven drying, and the yield further corrected for ash content. The yield of dry, ash-free hemicellulose is calculated as a percentage of the dry wood, being returned as "free hemicellulose," *i.e.* hemicellulose soluble in 4 % sodium hydroxide solution in the cold.

By this method, the losses of material resulting during the estimation can be reduced to negligible proportions. It is suggested that the free hemicellulose content of woody materials can be more accurately determined in this way than by other methods.

The wood residue, after removal of free hemicellulose, is treated with 300 cc. of boiling 4 % NaOH solution for 1 hour, the extract being filtered clear on calico as before. The residue is then repeatedly extracted with 150 cc. portions of boiling 4 % NaOH solution, till the filtered extract on cooling no longer gives any appreciable amount of precipitate on neutralising with glacial acetic acid and adding an equal volume of acetone.

The combined filtered extracts are neutralised and acetone added as above. The precipitate obtained is taken up to dryness in the usual way and is designated "combined hemicellulose."

It should be understood that the terms "free" and "combined" hemicellulose are employed relatively, rather than literally, to emphasise the more drastic treatment necessary to remove the latter.

The alkaline alcoholic filtrates reserved from above are rendered acid by the addition of glacial acetic acid, and 95 % alcohol may be further added to assist precipitation. The precipitate which separates out after prolonged standing is re-dissolved in 1 % NaOH, the solution is acidified, and alcohol

added to give a concentration of 75 %. The precipitate is allowed to settle, the liquid syphoned off and the product taken to dryness in the usual way.

This product, which appears to possess many properties in common with the hemicelluloses, is here described as "lignosaccharide," indicating its extraction with alcoholic sodium hydroxide, a solvent which readily extracts a portion of the lignin of woody tissues.

It is inconvenient to deduce the lignosaccharide content of the tissue from the yield obtained in this preparation, as the possibility of loss of product appears to be great. Consequently, the furfuraldehyde yield of the substance is determined, and its amount present in the wood calculated by the method shown below.

The results to this point are set out in Table II.

Table II.

Calculated on dry wood:	free hemicellulose	7·68 %
	combined hemicellulose	6·43
Furfuraldehyde yield from:	free hemicellulose	47·35 %
	combined hemicellulose	51·44
	lignosaccharide	38·10

The second stage of the estimation involves the treatment of 3–5 g. of the material with 200 cc. of 0·5 % ammonium oxalate solution at 85° for 24 hours. The solution is filtered, the residue washed with further ammonium oxalate solution, and the filtrate and washings are made up to 250 cc. The tissue residue is reserved. 100 cc. of the extract are treated for the estimation of total pectin by the method of Nanji and Norman [1928]. A second 100 cc. portion of the extract is evaporated gently to 50 cc., and 25 cc. of concentrated hydrochloric acid are added. The whole is washed into a distilling flask with 12 % HCl, and furfuraldehyde distillation carried out in the usual way. The furfuraldehyde arising is estimated and calculated to a percentage of the original dry wood.

This furfuraldehyde arises from pectin and from non-pectic substances simultaneously removed. The amount of pectin present being known, the non-pectic furfuraldehyde may be calculated by difference from the fact that 100 parts of calcium pectate yield 19·5 parts of furfuraldehyde.

The reserved tissue residue is extracted twice with 100 cc. portions of boiling 50 % alcohol containing 1 % of NaOH, the periods of extraction each being 1½ hours. The alcoholic filtrates are combined, and the tissue is finally washed with hot 50 % alcohol, the washings being added to the combined filtrates. To the extract is added glacial acetic acid so that the mixture remains just alkaline, the alcohol is evaporated off, and the total volume adjusted to 50 cc. 25 cc. of concentrated HCl are added, and the mixture is washed into a distilling flask, and furfuraldehyde again estimated and calculated to the dry wood.

Knowing now the amount of furfuraldehyde obtainable from the alcoholic

sodium hydroxide extract, and also the furfuraldehyde yield from the lignosaccharide, the percentage of this constituent in the tissue can be calculated. Thus, in the case of box-wood, the figures were 0·70 % of furfuraldehyde from the extract, and 38·10 % from the lignosaccharide, whence this wood contains 0·70/0·381 or 1·83 % of lignosaccharide.

If now a total furfuraldehyde determination is carried out on the wood itself, a complete furfuraldehyde balance sheet can be drawn up as shown in Table III.

Table III.

	% of dry wood
Furfuraldehyde from substances soluble in:	
(i) 0·5 % ammonium oxalate (*a*) pectin ...	0·08
(*b*) non-pectin	0·18
(ii) Alcoholic sodium hydroxide	0·70
Furfuraldehyde from:	
(iii) Free hemicellulose	3·64
(iv) Combined hemicellulose	3·31
(v) Other sources (by difference)	3·95
Total furfuraldehyde found directly	11·86

It was shown that the material remaining after removal of hemicelluloses from box-wood still retained a considerable amount of xylan.

Some 2 g. of the wood residue was treated with 72 % sulphuric acid according to the method of Monier-Williams [1921]. The dried products of hydrolysis were extracted with absolute alcohol. The bulk of the sugar produced was dissolved in this way and proved to be a mixture of glucose and xylose. Thus it yielded cadmium xylonobromide and a mixture of glucosazone, melting at 205°, and xylosazone melting at 155°. Other sugars could not be detected.

Consequently, the furfuraldehyde described as from "other sources" is converted to xylan, using the factor 1·55, the figure appearing as "other pentosans" in Table IV corresponding to this result.

Cellulose. This constituent was determined by chlorination, after a preliminary treatment of the tissue with 100 cc. of 1 % NaOH solution for 20 minutes. Chlorination was carried out in a Gooch crucible for periods of 20 and 15 minutes, followed by two further treatments of 10 minutes each. The resulting crude cellulose in every case gave large yields of furfuraldehyde on distillation with 12 % HCl. The yield of crude cellulose was therefore corrected, as suggested by Norman [1929], on the assumption that this furfuraldehyde is derived from xylan associated with the cellulose, the factor 1·55 being used for conversion of furfuraldehyde yield to xylan.

A further correction might conceivably be made assuming that the difference between furfuraldehyde yield from the crude cellulose, calculated back to the dry wood, and the furfuraldehyde from "other sources" of Table III represents hemicellulose not removed by chlorination. However, it is not unlikely that different portions of the hemicellulose complex might show

different degrees of resistance to removal by chlorination, and further correction on these lines was not attempted.

Lignin. It was recognised by Mehta [1925] that lignin estimation by alkaline treatment gave results which were very much lower than those obtained by the use of strong mineral acids.

Undoubtedly, some change is induced in lignin by acid treatment. Thus, lignin obtained by using 72 % sulphuric acid may still contain small amounts of carbohydrate material, while evidence has been obtained by Mehta that sulphonation may also take place. On the other hand, methoxyl and acetyl groups may be lost during the treatment, and on the whole it is not unlikely that the various errors tend to balance one another [*cf.* Euler, 1923].

In the present work, two methods of lignin estimation are used. The first is Mehta's method for free and combined lignin, these being returned separately. The second, carried out on wood previously extracted with alcohol, employs the 72 % H_2SO_4-HCl mixture of Schwalbe [1925] in the manner described by Norman [1929]. The probable relative value of the two methods is further discussed below.

Other constituents. Ash content is determined in the usual way. The loss in weight of the wood when exhaustively extracted with alcohol and ether successively is also found. If the acid method of lignin estimation is used, the value for total "alcohol-ether-soluble" is returned directly. If free and combined lignin are determined by alkaline treatment and returned separately, the value for free lignin must be subtracted from total alcohol-ether-soluble, and the difference returned as "free fat, pigment, *etc.*" The residue after alcohol-ether extraction is used for the determination of protein, the factor 6·25 being employed.

Finally, hot water-soluble substances are determined as described by Schorger [1926, p. 506]. If overlapping of results is to be avoided, "hot water-soluble" may not be returned directly. Thus, hot water dissolves some of the ash constituents, possibly protein, and some of the material soluble in alcohol and ether. Accordingly, these determinations are repeated on wood extracted with hot water, and the value for "hot water-soluble" is corrected. In some cases, the pectin soluble in hot water might also have to be taken into account, but it was not thought necessary to attempt this further correction in the case of woods.

Results of analysis.

The results obtained by the above methods of analysis are set out in Table IV. Two columns are given, allowing the values obtained by the two methods of lignin estimation to appear in separate columns, together with the correction where necessary of "alcohol-ether-soluble" to "free fat, pigment, *etc.*"

The summation of 87·78 % obtained when lignin is estimated by sodium hydroxide treatment is most unsatisfactory, since it is inconceivable that any constituent present in appreciable amount has escaped determination. Using

Table IV.

	% of dry wood	
Ash	1·22	1·22
Alcohol-ether-soluble ...	—	3·91
Free fat, pigment, *etc.* ...	2·05	—
Protein	2·81	2·81
Hot water-soluble (corrected)	0·71	0·71
Free lignin (NaOH)	1·86	—
Combined lignin (NaOH) ...	15·23	—
Lignin (H_2SO_4-HCl)	—	26·45
Pure cellulose	41·43	41·43
Calcium pectate number ...	0·41	0·41
Free hemicellulose	7·68	7·68
Combined hemicellulose ...	6·43	6·43
Lignosaccharide	1·83	1·83
Other pentosans (as xylan) ...	6·12	6·12
Total estimated	87·78	99·00

the acid method, on the other hand, the summation of 99·00 % represents a very satisfactory figure.

On this evidence, it is reasonable to conclude that figures for lignin content obtained by the alkali method cannot be accepted as reliable, and it may be mentioned that figures further supporting this view have been obtained with other woods.

B. Nature of the furfuraldehyde-yielding constituents of box-wood.

There remain to be discussed the results of a qualitative study of the furfuraldehyde-yielding constituents of the wood.

Water-soluble constituents. 100 g. of the sawdust were boiled for several hours with 1 litre of water. The filtered extract was cooled and concentrated to a bulk of 50 cc. 200 cc. of absolute alcohol were added, and after settling the supernatant liquor was poured off and reserved. The precipitate was heated with absolute alcohol to denature any protein present, and the residue was extracted with hot water, and the solution filtered. Four volumes of absolute alcohol were added to the filtrate, this treatment bringing down a very small amount of precipitate.

The product was readily soluble in hot water, the solution being non-reducing. It gave a blue-green colour with iodine solution, suggesting the presence of a little starch or amylo-hemicellulose [*cf.* Ling and Nanji, 1925]. On treating a little of the solution with Bial's orcinol reagent [see Browne, 1912, p. 382] the reaction for pentoses gave a negative result, but on boiling the solution a green colour developed, suggesting the presence of uronic anhydride. Possibly the substance responsible for this reaction is analogous to the ϵ-galactan of Trimble [1898] and of Schorger and Smith [1916], who isolated from western larch a substance yielding furfuraldehyde, but containing no pentosan. Their product was probably uronogalactan. The amount arising from box-wood was insufficient for examination.

The mother-liquor reserved from above was evaporated just to dryness *in vacuo* and the residue taken up in water. The solution, which had strong reducing properties, gave no coloration with iodine and was free from furfuraldehyde-yielding substances. Free simple sugars were evidently absent, since a crystalline osazone could not be obtained; the product instead was gelatinous, and its properties resembled those of phenylhydrazine derivatives prepared from the dextrinous products of starch conversions.

Lignosaccharide. The method of preparation of this substance has already been described, 100 g. of box-wood yielding 1·5 g. of lignosaccharide. The product was a fine white powder, somewhat soluble in cold water, completely in hot water to an opalescent, non-reducing solution. Lignosaccharide dissolves to a clear solution in 4 % NaOH solution and is not precipitated by Fehling's solution. It is precipitated, however, from the mixture on addition of acetone, thus behaving analogously to hemicelluloses B2 and C2 isolated from other sources.

Box-wood lignosaccharide gave 38·10 % of furfuraldehyde, and 6·65 % of carbon dioxide, the latter corresponding to a uronic anhydride content of 26·60 %.

This product showed a marked degree of resistance to hydrolysis. Thus, boiling for 2 hours with 5 % sulphuric acid produced a liquor in which free simple sugars could scarcely be detected.

Boiling for 7½ hours proved more successful, sugar being produced equivalent in amount to the hydrolysis of rather less than one-half of the lignosaccharide.

Two-thirds of this sugar was dissolved by absolute alcohol and proved to be chiefly xylose.

The sugar soluble in 80 % alcohol was treated with nitric acid for the preparation of mucic acid, but with negative results, so that the only constituents of the lignosaccharide which have been identified are uronic anhydride and anhydro-xylose.

Free hemicellulose. The wood residue remaining after removal of water-soluble substances, pectin, lignin, and lignosaccharide was treated with 4 % NaOH solution at room temperature, and the filtered extract was neutralised with glacial acetic acid and submitted to acetone fractionation. Only one fraction was obtained, corresponding to crude hemicellulose B, the yield from 100 g. of sawdust being 6·2 g. This was purified by copper treatment, the whole being precipitated from alkaline solution by Fehling's solution. Thus, the only free hemicellulose obtainable from box-wood sawdust is hemicellulose B1.

The purified product is a white, amorphous powder, easily soluble in hot water, the solution giving a faint opalescence on cooling. The solution is non-reducing and gives a faint greenish tinge with iodine. The hemicellulose is precipitated from aqueous solution by basic, but not by neutral, lead acetate. Three-quarter saturation with ammonium sulphate also gives a precipitate.

The hemicellulose is easily soluble in cold dilute alkali and in hot dilute mineral acids, the latter causing hydrolysis. The solution in 4 % NaOH solution gives for $c = 3{\cdot}00$ $[\alpha]_D^{20^\circ} - 62{\cdot}3^\circ$.

An aqueous solution of this hemicellulose is hydrolysed by the action of taka-diastase. After 24 hours, a precipitate is no longer given on rendering the solution alkaline and adding Fehling's solution, but the subsequent addition of acetone gives a thick precipitate. Iodine absorption corresponding to hydrolysis of 11 % of the hemicellulose takes place at this stage. At the end of 7 days, 40 % of the hemicellulose is apparently hydrolysed, and an equilibrium is reached at a little in excess of this value after 10 days. At the end of this time, the amount of precipitate given by the solution, either with acetone or a mixture of Fehling's solution and acetone, is extremely small. Free simple sugars cannot be detected in the final hydrolysis liquor.

The following analytical figures were obtained with hemicellulose B1, being calculated to a dry, ash-free basis:

Yield of furfuraldehyde	55·14 %
,, carbon dioxide	4·25 %
Hence, uronic anhydride	17·00 %

Since, as is shown below, the only sugar which can be detected amongst the products of hydrolysis of this substance is xylose, these figures may be interpreted as follows:

Furfuraldehyde from uronic anhydride	2·83 %
Hence, furfuraldehyde from xylan	52·31 %

The composition of the hemicellulose may now be calculated:

Uronic anhydride	17·00 %
Xylan	81·08 %
Total accounted for	98·08 %

This composition corresponds approximately to a molecule

$$\{(C_5H_8O_4)_6 \cdot (C_6H_8O_6)\}_n$$

which would yield 55·80 % of furfuraldehyde, and 4·55 % of carbon dioxide.

1·31 g. of the hemicellulose was hydrolysed with 75 cc. of 1 % sulphuric acid for 3½–4 hours. The sulphuric acid was neutralised with the calculated quantity of 0·25N barium hydroxide solution, and the filtered liquid, free from barium sulphate, titrated with 0·25N barium hydroxide till it remained just acid to phenolphthalein. 4·7 cc. were required to effect this, equivalent to $0{\cdot}176 \times 0{\cdot}25 \times 4{\cdot}7 = 0{\cdot}207$ g. of uronic anhydride, *i.e.* 15·7 % calculated on the hemicellulose. But the hemicellulose contains 17·0 % of uronic anhydride; hence, a practically quantitative liberation of the free carboxyl groups had been achieved. No insoluble barium salt was thrown down during this neutralisation of carboxyl groups.

The solution was taken just to dryness *in vacuo* and the residue extracted

several times with absolute alcohol. Xylose was isolated from the alcoholic extracts. The dry product weighed 0·55 g., corresponding to a yield of some 45 % of the theoretical. The xylose obtained gave $[\alpha]_D^{20°} + 18{\cdot}6°$, and the usual derivatives were prepared.

It has been shown in the case of the hemicelluloses of maize cobs [Preece, 1930] that 90 % of the theoretical amount of xylose can be obtained by the action of 1 % sulphuric acid. Hence, it appears fairly certain that acid of this strength does not cause a complete hydrolysis of box-wood hemicellulose; indeed, it seems to liberate only about one-half of the total possible amount of xylose.

Accordingly, the formula given above may be amended to

$$[(C_5H_8O_4)_3 \cdot \{(C_5H_8O_4)_3 \cdot (C_6H_8O_6)\}]_n$$

where one-half of the anhydro-xylose exists in a form of combination differing from that of the rest.

To test these suggestions further, a known weight of hemicellulose was treated with 1 % sulphuric acid, portions of the hydrolysis liquor being removed every half-hour, and the sugar present estimated iodimetrically by the method of Baker and Hulton [1920]. The results were calculated as apparent xylan and further expressed as a percentage of the hemicellulose hydrolysed. In a typical experiment, 1·473 g. of dry, ash-free hemicellulose was heated with 100 cc. of 1 % sulphuric acid.

Table V A.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
½	0·515	0·453	30·7
1	0·883	0·777	52·7
1½	1·024	0·901	61·2
2	1·065	0·938	63·7
2½	1·059	0·932	63·3

It is apparent from this table that an equilibrium is reached when there is a production of apparent xylose equivalent to the hydrolysis of some 64 % of the hemicellulose. However, the percentage of free xylose liberated in the first experiment described corresponds to a hydrolysis of xylan calculated on the hemicellulose of only 41 %. The difference between 64 % and 41 %, therefore, must be attributed to a liberation of free aldehydic groups, capable of reacting with alkaline iodine, but not present in the form of free xylose.

In continuation of the above hydrolysis, the volume of hydrolysis liquor

Table V B.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
3	0·799	0·703	79·5
3½	0·827	0·728	82·4
4	0·840	0·740	83·7
4½	0·875	0·771	87·2

remaining (60 cc.) was treated after $2\frac{3}{4}$ hours with 40 cc. of 10 % sulphuric acid, the resultant concentration of acid thus being 4·6 %, and 100 cc. of hydrolysis liquor now being equivalent to 0·883 g. of hemicellulose (see Table V B).

Thus, the increased concentration of acid caused an increase in the amount of hydrolysis, the rate, however, diminishing after the first few minutes.

Next the course of hydrolysis was followed, using 4·6 % sulphuric acid from the commencement. The equivalent of 0·7237 g. of dry, ash-free hemicellulose was treated with 50 cc. of 4·6 % sulphuric acid.

Table VI.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
$\frac{1}{2}$	0·473	0·416	57·5
1	0·652	0·574	79·3
$1\frac{1}{2}$	0·652	0·574	79·3
2	0·641	0·564	77·9
$2\frac{1}{2}$	0·613	0·540	74·6
3	0·673	0·592	81·8
$3\frac{1}{2}$	0·684	0·602	83·1
4	0·652	0·574	79·3

Here again the hydrolysis at first proceeds rapidly, but after 1 hour further action is slow, and eventually shows a falling off. This last effect is probably to be attributed to a destruction of uronic acid. Thus, O'Dwyer [1926] found that uronic acids tend to decompose during hydrolysis. This effect masks for a time the liberation of sugar, which, however, eventually re-asserts itself. It should be noted that the point at which the hydrolysis slackens—79·3 %—is almost exactly the same as the point of retardation found in the previous experiment—approximately 79·5 %.

Hydrolysis of the hemicellulose with an intermediate strength of sulphuric acid, namely 2·8 %, gave results agreeing closely with those obtained with 1 % acid, equilibrium being reached at an apparent hydrolysis of about 66 % of the hemicellulose.

Hydrolysis with either 1 % or 2·8 % acid, therefore, causes the rapid apparent hydrolysis of 64–66 % of the hemicellulose, subsequent action being absent or very slow. 4·6 % acid causes the rapid apparent hydrolysis of approximately 80 % of the hemicellulose, the action proceeding slowly beyond this point, when its effect may be at least partially masked by a destruction of uronic acid.

The results obtained may be best interpreted as follows. Suppose the structure of the hemicellulose is represented by the formula

$$\underset{\text{I}}{\{(C_5H_8O_4)_3} . \underset{\text{II}}{(C_5H_8O_4)_2} . \underset{\text{III}}{(C_5H_8O_4 . C_6H_8O_6)\}_n}.$$

It is to be assumed that the following changes take place. I is rapidly hydrolysed by 1 % H_2SO_4 to free xylose. II is converted to a disaccharide of

the form $C_5H_9O_4.O.C_5H_9O_4$ or, since it contains a free aldehydic group, $CHO.C_4H_8O_3.O.C_5H_9O_4$. III is converted to a similar disaccharide derivative, containing one free aldehydic group, and one free carboxyl group. This would account for the liberation of the total acidity without formation of free uronic acid. It appears likely that the uronic acid concerned is glycuronic acid, and indeed free glycuronic acid could not be detected amongst the products of hydrolysis of the hemicellulose by 1 % acid.

The suggestion that a disaccharide derivative of the type of a xylosido-uronic acid is formed may be compared with the observation of Butler and Cretcher [1929] that hydrolysis of arabic acid, obtained from gum arabic, with 2 % sulphuric acid yields *d*-galactosido-*d*-glycuronic acid.

Hydrolysis of the hemicellulose in this way would result in the liberation of three molecules of xylose, one free carboxyl group, and two free additional .CHO groups.

4·6 % acid causes a further liberation of .CHO groups. Suppose the portion III of the molecule is, along with I, readily hydrolysed to its components by this stronger acid, while II is still only slowly attacked. Then a total of 4 molecules of free xylose, one of uronic acid (now susceptible to the destructive action of the mineral acid), and one additonal free .CHO group would be liberated.

Assuming that 90 % of the free xylose produced is isolated as such, the theoretical yields on the foregoing assumptions and the yields actually obtained are given in Table VII.

Table VII.

	1 % H_2SO_4		4·6 % H_2SO_4	
	Free xylose from 1 g. of hemicellulose	Total apparent hydrolysis*	Free xylose from 1 g. of hemicellulose	Total apparent hydrolysis*
Theory	0·42 g.	68 %	0·56 g.	82 %
Actual	0·42	65	0·52	80

* Calculated as a percentage of xylan on the hemicellulose.

Thus, there appears to be good evidence for assuming that the hemicellulose is a polymerised substance of the formula given above.

Combined hemicelluloses. The tissue residue remaining after removal of free hemicelluloses was extracted several times with boiling 4 % NaOH solution, the extracts obtained being filtered and combined. Neutralisation of the cooled extracts with glacial acetic acid caused a precipitation of combined hemicellulose fraction A. This was centrifuged off, and half a volume of acetone was added to the filtered centrifugate. Fraction B was thus precipitated. The filtrate from this gave a further small amount of precipitate on adding more acetone, but the yield of fraction C arising in this way was too small for examination.

Heated with water, fraction A gives a silky suspension, which is non-reducing and gives a greenish tinge with iodine. This hemicellulose becomes

soluble in cold 4 % NaOH, and the bulk of it is precipitated from alkaline solution on addition of Fehling's solution. A separation on these lines into its components A1 and A2 was not attempted, owing to the very small amount of A2 which would thus have been obtained.

Fraction B is soluble in hot water; the solution gives a pale green colour with iodine and is non-reducing. This fraction also dissolves in cold 4 % NaOH solution and is partially re-precipitated on addition of Fehling's solution. Again, a separation was not made.

The usual analytical data were obtained, as shown in the following table.

Table VIII.

Hemicellulose fraction	A	B
Furfuraldehyde yield	36·57 %	48·29 %
Carbon dioxide yield	3·22	4·33
Uronic anhydride content	12·88	17·32

In each case hydrolysis was carried out by boiling for 2 hours with 5 % sulphuric acid. After removal of sulphuric acid, the hydrolysis liquid was evaporated just to dryness *in vacuo*, and the sugar extracted successively with absolute and 80 % alcohol. Several extractions were made with alcohol of each strength. The filtered extracts were worked up for the preparation of dry sugar fractions.

Some 65 % of the sugar obtained from combined hemicellulose A was dissolved by absolute alcohol. This product had $[\alpha]_D^{20^\circ} + 17^\circ$ and represented an impure sample of xylose. The sugar dissolving in 80 % alcohol gave $[\alpha]_D^{20^\circ} + 26^\circ$, but sugars other than xylose could not be detected even in this fraction.

In the case of combined hemicellulose B also the bulk of the sugar produced on hydrolysis was dissolved by absolute alcohol. The sugar of this fraction was again impure xylose.

80 % alcohol dissolved sugar having $[\alpha]_D^{20^\circ} + 49^\circ$. The osazone prepared from the sugar of this fraction was a mixture of indefinite character and low melting-point, but certainly contained much xylosazone. An attempt to prepare cadmium xylonobromide was successful. From the figure quoted and the fact that xylose is present to an appreciable extent in the sugar of this fraction, it would seem that the second sugar of this fraction is one with a high specific rotation. However, mucic acid tests gave negative results, while the absence of arabinose from the hydrolysis products is indicated by the low specific rotation of the sugar of the absolute alcohol-soluble fraction, namely $[\alpha]_D^{20^\circ} + 20^\circ$. Thus the nature of the second sugar must remain at present undetermined.

The tissue residue. The wood residue remaining after removal of combined hemicelluloses was examined. Attempted estimations of mannan and galactan were carried out by the methods described by Schorger [1926, pp. 537, 538], but both these substances were found to be absent. As has been shown above,

the pentosan constituent of the residue is xylan. It was also found that boiling dilute acid—5 % sulphuric acid—had but little hydrolytic effect on the wood residue.

Discussion.

Since the amount of pectin present in the wood is 0·41 %, the proportion of anhydro-arabinose is less than 0·1 %. Anhydro-xylose, on the other hand, amounts to approximately 17 % of the wood, while the total uronic anhydride content is of the order of 3 %. Unidentified anhydro-hexose or anhydro-methylpentose constituents of the hemicelluloses and lignosaccharide account for some 2 % of the wood.

It is apparent from the work described that there is little difference in nature between the combined hemicelluloses isolated from box-wood and the free hemicelluloses from box-wood and from the other sources dealt with previously [Norris and Preece, 1930; Preece, 1930, 1931]. The lignosaccharide, though more resistant to hydrolysis, is also of a similar nature. These constituents, consequently, can be grouped together as "structural hemicelluloses," the name "hemicellulose" being retained in preference to that of "polyuronide" suggested by Candlin and Schryver [1928].

It is desirable to distinguish between the hemicelluloses and the xylan of the wood residue. This xylan resists extraction by the relatively drastic action of boiling 4 % NaOH solution and is also resistant to the hydrolytic action of boiling dilute mineral acids. In short, its properties approach more nearly those of cellulose than those of the hemicelluloses. Accordingly, it is proposed to make such a distinction by the re-introduction of the term "para-xylan" for this resistant form of xylan. Thus, the final wood residue may be said to consist of cellulose and para-xylan, together with some unextracted lignin.

Although it has been found possible to assign a comparatively simple formula to the free hemicellulose of box-wood, it is not claimed that the fractions described in this and previous communications represent individual hemicelluloses [see also Preece, 1930]. It has been shown by O'Dwyer [1928] that the complexity of a hemicellulose seems to vary with the age of the wood from which it is obtained. If change in complexity is regularly taking place, this must lead to the presence of a large number of true hemicellulose units of different composition. Since the present method of separation of free hemicelluloses cannot give more than six fractions, it would seem that these fractions must, generally speaking, be mixtures, representing aggregates of molecules of similar physical properties.

A similar argument might, it may be supposed, be applied to lignosaccharide and combined hemicelluloses. An inter-relationship between these three subdivisions of the structural hemicelluloses might conceivably, if shown to exist, lead to a revision of the above view.

SUMMARY.

1. A method of analysis of woody tissues giving a very satisfactory summation is described, various types of furfuraldehyde-yielding substance being estimated separately.

2. The alkali method for lignin estimation gives results which cannot be accepted.

3. Free pentoses are absent from box-wood, but hot water extracts some non-pentosan furfuraldehyde-yielding substance.

4. Lignosaccharide and free and combined hemicelluloses have been isolated from box-wood, and their nature examined. Xylose and uronic acid arise on hydrolysis of each of these substances.

5. The composition of the free hemicellulose is completely accounted for as urono-xylan of relatively simple constitution. The hydrolysis of this substance has been closely studied, and different parts of the molecule shown to possess different degrees of resistance to hydrolysis by dilute sulphuric acid.

6. The wood residue remaining after extraction of the above constituents consists chiefly of cellulose, para-xylan, and some unextracted lignin.

The author wishes to express his indebtedness to Prof. A. R. Ling and Dr F. W. Norris for the interest they have shown in this work.

REFERENCES.

Baker and Hulton (1920). *Biochem. J.* **14**, 754.
Browne (1912). Handbook of sugar analysis. (New York.)
Butler and Cretcher (1929). *J. Amer. Chem. Soc.* **51**, 1519.
Candlin and Schryver (1928). *Proc. Roy. Soc. Lond.* B **103**, 365.
Cross, Bevan and Smith (1895). *Ber. deutsch. chem. Ges.* **28**, 2604.
Dore (1920). *J. Ind. Eng. Chem.* **12**, 476, 984.
Euler (1923). *Cellulosechemie*, **4**, 1.
Konig and Becker (1919). *Z. angew. Chem.* **32**, 155.
Ling and Nanji (1925). *J. Chem. Soc.* **127**, 652.
Mehta (1925). *Biochem. J.* **19**, 958.
Monier-Williams (1921). *J. Chem. Soc.* **119**, 803.
Nanji and Norman (1928). *Biochem. J.* **22**, 596.
—— Paton and Ling (1925). *J. Soc. Chem. Ind.* **44**, 253 T.
Norman (1929). *Biochem. J.* **23**, 1353.
Norris and Preece (1930). *Biochem. J.* **24**, 59.
O'Dwyer (1926). *Biochem. J.* **20**, 656.
—— (1928). *Biochem. J.* **22**, 381.
Preece (1930). *Biochem. J.* **24**, 973.
—— (1931). *J. Inst. Brew.* **37**, 409.
Schorger (1926). Chemistry of cellulose and wood. (New York.)
—— and Smith (1916). *J. Ind. Eng. Chem.* **8**, 494.
Schwalbe (1925). *Papier Fabrk.* **23**, 174.
Trimble (1898). *Amer. J. Pharm.* **70**, 152.

CXLIV. THE OCCURRENCE OF PYRUVIC ACID AND METHYLGLYOXAL IN MUSCLE METABOLISM.

By EDWIN MARTIN CASE[1] and ROBERT PERCIVAL COOK[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 2nd, 1931.*)

That the metabolic changes which occur in muscle are of profound complexity is evident. This work was undertaken with the object of studying some of the intermediary mechanisms of muscle carbohydrate metabolism by means of the use of reagents which would remove from the system some product which is only transitorily formed normally. The method is essentially the same as that employed by Neuberg in his study of the mechanisms involved in alcoholic fermentation. The fixative of which we have availed ourselves, namely sodium sulphite, is the same as that used by Neuberg in what constitutes his second form of fermentation, in which acetaldehyde is "fixed" as the bisulphite compound. It is well known that substances of an aldehydic or ketonic nature possess the property of forming addition compounds with sodium bisulphite. The conditions that determine the stability or otherwise of these compounds to further metabolic change have not, however, been worked out. In the conditions under which we have worked, pyruvic acid and methylglyoxal will form with bisulphite compounds that are relatively stable in this regard.

Neuberg and Reinfurth [1920] have shown that in the case of yeast the pyruvate-bisulphite complex is decarboxylated to acetaldehyde. Cook [1930] has confirmed this observation for yeast but has noted that *B. coli communis*, for example, is unable to bring about this decarboxylation. It was also noted that if oxidations of various substrates were carried out in the presence of sulphite, the sulphite served as a very effective fixative for any pyruvic acid that was formed. The method of sulphite fixation also furnishes evidence as to the occurrence of other aldehydic or ketonic compounds during the course of metabolism.

It may be, and often is, urged that the fixation method is liable to give a false impression of the chemical events taking place in that it tends to endow subsidiary reactions with a spurious prominence. It will be shown, however, that as regards the carbohydrate metabolism of muscle it is possible, if sufficient care be taken, to identify pyruvic acid in normal muscle in the absence of any fixatives, and in amounts commensurate with those found when a fixative is

[1] Beit Memorial Research Fellow.

present. It is only to be expected, nevertheless, that under normal conditions the pyruvic acid, if of importance in metabolism, would quickly disappear. As will be shown later, this disappearance is indeed extremely rapid in the normal muscle.

Although there is no doubt that the presence of sulphite does affect the normal balance of reactions, we are of the opinion that this method of fixation affords useful indications concerning some of the chemical events associated with the metabolism of muscle.

The oxidation of lactate by muscle tissue.

It was postulated by Meyerhof, and the view commands general acceptance, that part of the lactic acid in muscle is oxidised in order to provide energy for the resynthesis of the remainder to carbohydrate. This, taken in conjunction with the fact that lactic acid is dehydrogenated to pyruvic acid by muscle tissue in the presence of methylene blue [Hahn, Fischbach and Haarman, 1928] possibly indicates that at least part of the lactic acid would, under aerobic conditions, with atmospheric oxygen as the hydrogen acceptor, be oxidised *via* pyruvic acid. In the instance of the oxidation of lactate by *B. coli*, pyruvate may be fixed by the use of sulphite, the oxidation (measured as oxygen uptake) corresponding to 72 % transformation of lactate to pyruvate [Cook, 1930]. It was hoped that by the employment of similar methods with muscle, any pyruvic acid which was formed would be made evident by the presence of the sulphite.

As a preliminary it was desirable to investigate the effect of the addition of lactate on the normal respiration of muscle. The respiration was measured as the oxygen uptake in the Barcroft differential manometer. The left-hand pot contained 1·5 cc. frog Ringer solution, 1·0 cc. phosphate buffer (p_H 7·2) and 0·5 cc. water. The right-hand pot contained 1·5 cc. Ringer solution, 1·0 cc. buffer and 0·5 cc. water or $M/10$ lactate solution, together with a weighed amount of frog muscle. Each of the small central pots contained a roll of filter-paper soaked in 6 % potassium hydroxide to absorb CO_2.

It is evident that the metabolism of the muscle is affected by the physiological conditions of the tissue, *i.e.* is altered by circumstances such as chopping, mincing or washing. Some of the results obtained are shown in Table I.

It will be seen that there appears to be a slightly greater oxygen uptake with intact muscle on the addition of a solution of sodium lactate (Exp. 1). On the other hand the addition of lactate to unwashed chopped muscle has little effect on the oxygen uptake (Exp. 3). There is a slightly increased uptake upon the addition of lactate to washed chopped muscle (Exp. 5), but it is doubted if this is significant. The addition of sulphite causes a marked decrease in the oxygen uptake both with intact and chopped muscle (Exps. 2 and 4).

The initial velocities of oxidation of the intact gastrocnemii were the same in the two instances, but after 3 hours a small but appreciable difference was noted in the rate of oxygen uptake of the control as compared with the muscle

Table I.

				Controls Ringer + phosphate p_H 7·2				Ringer + phosphate + $M/60$ Na_2SO_3 p_H 7·2			
						+ $M/60$ lactate				+ $M/60$ lactate	
Remarks	No. of exp.	Temp. (°)	Duration of exp. (hrs.)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)
Intact (gastrocnemii)	1	24	4·75	0·79	208·5	0·76	230·8	—	—	—	—
	2	25	4	0·49	125	—	—	0·56	31·5	0·53	60·9
Chopped unwashed	3	23·5	2·5	0·5	336	0·5	340	—	—	—	—
	4	19·5	3·5	—	—	0·5	238	—	—	0·5	74·1
Chopped washed	5	23·5	4	0·5	61·6	0·5	73·2	—	—	—	—

to which lactate had been added. It is evident therefore that some oxidation of the added lactate has taken place.

It is realised that in the oxidation of lactate by muscle no increase in velocity would be expected in either intact or unwashed chopped muscles, as the enzymes dealing with the oxidation of lactate would be saturated with this substance; hence no difference in velocity could be expected as long as this amount remains in excess. None of our experiments was of more than 6 hours' duration and in no case was the oxidation in the presence of lactate very much greater than that in its absence. It is clear that under our conditions at least, the oxidation of lactate by muscle is a relatively slow process.

The muscles in Exps. 2 and 4 were removed from the pots after 4 hours, rapidly ground with sand and filtered through muslin. Tests for pyruvate were then carried out by the method of Simon and Piaux [1924]. (A description of this is given later.) Slight positive reactions were given by the intact muscle and unwashed chopped muscle in the presence of sulphite, the controls in which no sulphite was present being definitely negative.

Some estimations were carried out on the disappearance of lactic acid in the systems both with and without the addition of sulphite. The results obtained were however rather indefinite, but it appears that the oxidation of lactate constitutes only a small percentage of the total respiration. Under the conditions described the Friedemann, Cotonio and Shaffer [1927] method for the determination of lactic acid is rather unsatisfactory. It is hoped to give further details of the relation of pyruvic acid formation to lactic acid oxidation in a later paper.

Methods for the detection and estimation of compounds formed.

As an indication of the formation of pyruvic acid we have generally employed the test of Simon and Piaux [1924]. Considerable caution must, of course, always be exercised in using a colour reaction as the sole evidence of formation of any particular compound. As will appear later we have not relied exclusively upon colour tests, and we have invariably found that the indications afforded by other methods have run closely parallel with those obtained

by the Simon and Piaux reaction. As a qualitative indication of the presence of pyruvic acid this test is carried out as follows. About 3 cc. of the material to be examined are placed in a test-tube and ammonium sulphate is added in such quantity that a layer rests at the bottom of the tube. Crystals of sodium nitroprusside are sprinkled on this layer and an equal volume of ammonia (sp. gr. 0·880) is then added without shaking. In the presence of filtrates prepared from muscle a pink colour at first makes its appearance. This reaction is due to the .SH compounds present, but rapidly fades and does not interfere with the pyruvic acid reaction. This latter reaction appears about half an hour after the commencement of the test and consists in a blue or greenish-blue colour, which develops in the neighbourhood of the nitroprusside crystals, and if the concentration of pyruvate be sufficiently great spreads throughout the solution. The colour increases in intensity for about 2 hours, after which it begins to fade, and with higher concentrations of pyruvate a red colour makes its appearance after the lapse of about 12 hours. Although nitroprusside and alkali hydroxides give various colour reactions with numerous aldehydes and ketones and certain unsaturated sulphur derivatives, we are not aware of any substance of likely physiological importance, other than pyruvic acid, which gives with nitroprusside and ammonia the characteristic blue-green colour; and we are of the opinion that this test is a reliable criterion for the presence of pyruvic acid. We have tested the reaction with a solution of methylglyoxal but have not obtained a positive result. With nitroprusside and alkali hydroxide methylglyoxal gives a red colour which is however insufficiently specific to constitute a useful test.

Needless to say, any satisfactory proof of the formation of a compound must involve either the isolation of the actual compound or the isolation of some convenient derivative. The first alternative is in the present case obviously rather difficult to undertake. For the purposes of identification we have used *p*-nitrophenylhydrazine and 2 : 4-dinitrophenylhydrazine. The method used in the earlier experiments was to precipitate the proteins with trichloroacetic acid and then to add drop by drop a freshly prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. This has the disadvantages that in the first place considerable concentration of the material under test is necessary, and that in the second place precipitation of the reagent itself is apt to occur when the acid solution is added even to water alone. Hence the precipitates were frequently contaminated with considerable amounts of unchanged reagent, which tended to interfere with the subsequent colour reactions with alcoholic potassium hydroxide. Nevertheless, since we at first used this substance, we have prepared the derivatives of pyruvic acid and methylglyoxal from these compounds themselves, and have for convenience incorporated some of their properties in Table II. For the separation of these derivatives we have followed the method described fully by Dakin and Dudley [1913].

It was found that far better results were obtained by using 2 : 4-dinitro-

phenylhydrazine, which is very easily prepared by the method given by Allen [1930]. This substance is used in the form of a solution of 0·5 g. in 30 cc. 2*N* hydrochloric acid. No precipitation of the reagent occurs on adding to water. The method employed is essentially the same as that described by Neuberg and Kobel [1930]. The characteristics of the derivatives of this compound are also given in Table II. The acetaldehyde hydrazones are included in addition, in view of the fact that Neuberg and Gottschalk [1925] have reported the occurrence of acetaldehyde in the course of muscle metabolism in the presence of sulphite. As will be mentioned later, we have not been able to demonstrate the presence of acetaldehyde in any amount comparable with that of pyruvic acid or methylglyoxal in any of our experiments. We have employed Rimini's [1904] test for acetaldehyde, but the reaction obtained would correspond to the presence of only extremely small amounts of this substance.

Table II.

Reagent	Pyruvic acid hydrazone			Methylglyoxal osazone			Acetaldehyde hydrazone		
	Colour of precipitate	Reaction with alcoholic potash (6 %)	M.P.	Colour of precipitate	Reaction with alcoholic potash	M.P.	Colour of precipitate	Reaction with alcoholic potash	M.P.
p-Nitrophenylhydrazine	Yellow	Cherry-red	223–225°	Red*	Deep blue	302–304°	Yellow	Brown-red	128°
2 : 4-Dinitrophenylhydrazine	Lemon-yellow	Deep red	214°	Light red†	Blue-violet	298°	Yellow	Deep red	147°

* Recrystallised from nitrobenzene and heated for some time at 140°.
† Recrystallised once from pyridine and once from nitrobenzene.

A useful confirmatory test, though in itself anything but conclusive, for the presence of pyruvic acid and methylglyoxal is the iodoform reaction. Iodoform is rapidly produced in the cold by both methylglyoxal and pyruvic acid on the addition of alkali and iodine.

We have also employed the capacity of these compounds for binding sulphite as a method of estimating roughly the amounts formed. The procedure is described by Cook [1930]. It is possible by this method to estimate both the pyruvic acid and the methylglyoxal in a single sample. The total bound sulphite is estimated, the solution then being made acid and the methylglyoxal distilled off into sulphite and titrated in the distillate.

Experiments on rabbit muscle.

When working with frog muscle one is necessarily limited with respect to the amount of muscle it is possible to employ and hence the quantity of metabolic products which it is possible to detect or isolate. In order to overcome this limitation it was thought desirable to attempt to use rabbit muscle. The use of mammalian muscle for experiments of this nature is attended with several disadvantages, prominent among which are its comparatively rapid

deterioration when excised, either at the temperature of the animal or at lower temperatures, and the impossibility of ensuring that the tissue shall be permeated by any constituent of the fluid in which it may be immersed. We therefore decided to try minced rabbit muscle, although from the outset we realised fully the unphysiological condition of the tissue, and the fact that mincing or chopping destroys the power of the muscle to resynthesise carbohydrate.

The muscle was taken from the hind limbs and back of a rabbit killed by a blow on the head and bled from the heart. It was immediately minced, weighed, and apportioned out into beakers containing the various solutions prepared to receive it. After thorough mixing and disintegration of the chopped mass, the contents of the beakers were thoroughly aerated with oxygen, bubbled through for the duration of the experiment, which as a rule lasted for several hours at room temperature. At intervals nitroprusside tests were performed on about 3 cc. of the mixed tissue and fluid.

The details of the first of these experiments are as follows. Beaker A (control), 250 cc. phosphate buffer (p_H 7·2), 100 g. minced muscle; Beaker B (with sulphite), 242·5 cc. phosphate buffer, 7·5 cc. *M* Na_2SO_3, 100 g. minced muscle. These were oxygenated at room temperature for $3\frac{1}{2}$ hours. At the end of this time the contents of both beakers were strained through muslin and deproteinised by the addition in each case of 9 cc. of 50 % trichloroacetic acid. After filtering, the fluids were concentrated on a water-bath to about 20 cc. When cool, a few drops of an acetic acid solution of *p*-nitrophenylhydrazine were added to each, and the precipitates obtained were filtered off and washed. It was noted that the yield of solid from B was considerably the greater of the two.

The results are embodied in Table III.

Table III.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A (control)	B (with sulphite)
15	+ + +	+ +
40	+ +	+ + + +
65	+ +	+ + + +
210	+	+ + + +

Colour reactions of hydrazones with alcoholic potassium hydroxide.

A	B
Dirty bluish brown, similar to the colour obtained with excess of reagent	Strong deep red. Indicative of pyruvic acid

It is obvious that although towards the beginning of the experiment pyruvic acid was present to about an equal extent in both mixtures, it rapidly disappeared in the control, but on the other hand increased in concentration in the presence of the fixative.

Although the occurrence of pyruvic acid in minced rabbit muscle was thus

demonstrated, there was of course nothing to indicate that its presence was in any way dependent upon any oxidative process, in spite of the strongly aerobic conditions. The next step was to repeat the experiment under both aerobic and anaerobic conditions. To this end mixtures were set up as follows:

A. 50 g. minced muscle, 200 cc. buffer (p_H 7·2)	Aerobic	C. As A	Anaerobic
B. 50 g. minced muscle, 194 cc. buffer, 6 cc. M Na_2SO_3		D. As B	

A and B were oxygenated as before in beakers, while C and D were placed, together with 1 cc. of $M/10$ potassium cyanide solution, in Büchner flasks, which were evacuated. Nitroprusside tests were done from time to time over a period of about 4 hours. At the end of this time, 5 cc. of each mixture were ground with sand and the filtrates used for iodine titrations for the purpose of getting a rough idea of the relative amounts of bound sulphite. (In the cases of A and C a little sulphite was added to the liquid before titration.) The main bulk of the contents of the vessels was filtered through muslin and made up to 250 cc., including the addition of 20 cc. of 50 % trichloroacetic acid. The filtrates from this were concentrated to about 25 cc. and after cooling, 4 cc. of 2 : 4-dinitrophenylhydrazine solution in HCl added to each.

Table IV gives the complete results of this experiment.

Table IV.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A	B	C	D
40	−	−	−	+
175	+ −	+	−	+ +
240	−	+ +	−	+ + −
300	−	+ +	+ −	+ +

Iodine titrations for bound sulphite.

(5 cc. filtrate before deproteinisation was used; strength of iodine was 0·008 N.)

A	B	C	D
0·15 cc.	1·05 cc.	0·10 cc.	1·10 cc.

Extent to which precipitation occurred on addition of reagent.

	A	B	C	D
10 mins. after adding reagent	−	+ +	+ −	+ +
90 mins. after adding reagent	+	+ + +	+ +	+ + +

Colour reactions of hydrazones with alcoholic potash.

A	B	C	D
Blue-violet. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid	Blue-violet. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid

Here again it is seen that, in the presence of sulphite, pyruvic acid accumulates in quantities much greater than are found in the absence of the fixative. The colours given by the precipitates with 2 : 4-dinitrophenylhydrazine correspond exactly with the distribution of pyruvic acid as evinced by the nitroprusside tests. It is interesting also to note that in the absence of sulphite

methylglyoxal seems to preponderate over pyruvic acid. But the most important fact brought to light by this experiment is that in the presence of sulphite, the imposition of either aerobic or anaerobic conditions seems to make no difference to the appearance of pyruvic acid. This is of great significance, indicating as it does that the occurrence of pyruvic acid is not necessarily associated with oxidative processes, since these are excluded in C and D.

One explanation that suggests itself is that pyruvic acid (and methylglyoxal) may be intermediaries in the formation of lactic acid; indeed it seems difficult to place any other interpretation upon the results. If this is so, it should be possible to detect pyruvic acid among the substances present in an aqueous muscle extract during glycolysis. The next experiment to be done was then the following.

A. 100 cc. muscle extract
5 cc. phosphate buffer (p_H 7·2) (ten times strength)
3 cc. *M* Na_2SO_3
15 cc. 2 % glycogen
5 cc. water
} Oxygenated

B. 100 cc. muscle extract
5 cc. buffer (× 10)
3 cc. *M* Na_2SO_3
15 cc. water
5 cc. *M*/30 KCN

C. 100 cc. muscle extract
5 cc. buffer (× 10)
3 cc. *M* Na_2SO_3
15 cc. 2 % glycogen
5 cc. *M*/30 KCN
} Anaerobic

The muscle extract was prepared from rabbit muscle in the manner described in a former paper [Case, 1929]. Nitroprusside tests were performed as usual at intervals, and at the conclusion all the fluids were precipitated with 10 cc. of 50 % trichloroacetic acid, filtered, concentrated and treated with *p*-nitrophenylhydrazine. Table V sets forth the results.

Table V.

Nitroprusside reactions.

Time from commencement of exp. (mins.)	A	B	C
3	–	–	–
60	–	–	–
120	–	+	+
345	+	+	+ + +

Colour reactions of p-*nitrophenylhydrazones* (*-osazones*) *with alcoholic potassium hydroxide.*

A	B	C
Purplish-blue. Indicative of methylglyoxal with slight admixture of pyruvic acid	Deep blue. Indicative of methylglyoxal	Deep red, with very slight purplish tinge. Indicative of pyruvic acid, slightly contaminated with methylglyoxal

In this experiment, we find once more an appearance of pyruvic acid under anaerobic conditions, though not so marked quantitatively as in the case where the minced tissue was present in the system. Moreover, the addition of glycogen (in C) has caused a rise in the amount of pyruvic acid formed. As up to the present no attempt has been made to repeat this, we hesitate to

place much reliance on the result; but the fact, if confirmed, that added glycogen does have this effect seems to support the possibility that pyruvic acid is concerned in the process of glycolysis.

An experiment was now carried out with the object of ascertaining whether the presence of added lactate in the solution would have any similar effect. If pyruvic acid is being formed during the oxidation of lactic acid it might be expected that this would be the case.

A. 70 g. minced muscle
12·5 cc. buffer (× 10)
3·25 cc. *M* Na_2SO_3
Water to 125 cc.

B. 70 g. minced muscle
12·5 cc. buffer (× 10)
3·25 cc. *M* Na_2SO_3
10 cc. *M*/10 sodium lactate
Water to 125 cc.

} Aerobic, at room temperature

C. As A
D. As B
} Aerobic, in incubator at 35°

Table VI.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A	B	C	D
0	+ +	+ +	+ +	+ +
110	+ + +	+ + +	+ + +	+ + +
180	+ +	+ +	+ +	+ +
240	+ +	+	+	+

No hydrazones were prepared in this case, as the filtrates were used for other purposes, but it is obvious that the addition of lactate has not brought about any increase in amount of pyruvic acid, such as was observed when glycogen was added.

Up to this point attention has been concentrated mainly upon the detection of pyruvic acid. The demonstration of the presence of methylglyoxal is a more difficult matter, apart from the isolation of the osazone, since no rapid test exists for its identification in solution, corresponding to the Simon and Piaux reaction for pyruvic acid. In order to effect a convenient separation of methylglyoxal from pyruvic acid we resorted in several experiments to the procedure of which the ensuing is a typical example.

A flask was set up containing 25 cc. buffer (× 10), 6·5 cc. *M* Na_2SO_3, water to 250 cc. and 100 g. of minced muscle. This was allowed to incubate at room temperature for 5 hours. The nitroprusside reaction was then positive for pyruvic acid. The mixture was filtered through muslin, precipitated with trichloroacetic acid and again filtered. 20 cc. of 10 % sulphuric acid were added, and the whole of the fluid was distilled for an hour, the distillate being

Table VII.

	Product of interaction of 2 : 4-dinitrophenylhydrazine with	
	Residue in flask	Distillate
Colour of precipitate	Light yellow	Orange-red
Colour reaction with alcoholic potash	Deep red	Blue-violet

received in 10 cc. of 1 % sodium bisulphite cooled in ice. After cooling, both the residue in the flask and the distillate were treated with 2 : 4-dinitrophenylhydrazine (Table VII).

This shows that methylglyoxal has been distilled over, leaving pyruvic acid behind.

Attempts to demonstrate the presence of acetaldehyde in the distillate, where if formed it would be found, met with no success. The Rimini test was negative, and the characteristics of the hydrazones were not consistent with the presence of acetaldehyde (see Table II). If present at all, it can be only in very small amounts as compared with the amounts of methylglyoxal and pyruvic acid. Nitroprusside tests were strongly positive for the residue in the distilling flask, and negative for the distillate. Both fractions gave strongly positive iodoform reactions. In the case of a control experiment in which no sulphite was added, practically no nitroprusside test was given, and the iodoform reaction was only faintly positive.

It was considered desirable to perform a large-scale experiment with a view to isolating the derivatives of the products in sufficient quantity to carry out not only colour reactions but also melting-point determinations, in order to place their identification beyond question. The mixture employed consisted of 50 cc. phosphate buffer (× 10), 25 cc. *M* Na_2SO_3, water to 1 litre, 463 g. minced muscle. This was incubated at room temperature for 5 hours, at the end of which period a very strong nitroprusside test for pyruvic acid was given. The mixture was filtered through linen, using a press. Just over 1000 cc. of filtrate was obtained, and this was deproteinised with 100 cc. of 50 % trichloroacetic acid and filtered. The clear filtrate was again tested with nitroprusside and still gave a strongly positive reaction. It was then concentrated at about 40° *in vacuo* to a volume of 150 cc., more sulphite being added to ensure that the methylglyoxal and pyruvic acid should remain bound. After cooling, 40 cc. of 2 : 4-dinitrophenylhydrazine in HCl were added, the mixture was heated for a few minutes on a boiling water-bath and allowed to stand for some hours[1]. A fairly bulky orange precipitate was obtained, which was filtered off and washed with a little 0·5*N* HCl. The precipitate was treated with warm 25 % sodium carbonate, which dissolved the pyruvic acid derivative giving a dark-brown solution [Neuberg and Kobel, 1930]. On acidifying this solution with HCl, the hydrazone of pyruvic acid separated out as a lemon-yellow solid, which was further purified by re-solution in sodium carbonate and re-precipitation with acid, and finally by crystallisation from ethyl acetate.

Meanwhile the residue from the first sodium carbonate extraction, containing the methylglyoxal derivative, was washed once more with hot sodium carbonate to remove traces of the pyruvic compound, then with hot 2*N* HCl, and lastly with boiling water. It was then dried with a small quantity of

[1] When sulphite is present, this heating is frequently essential in order that hydrolysis of the sulphite-complex may be effected. If it be omitted, precipitation may be delayed or even suspended altogether.

alcohol and ether and recrystallised from pyridine; a dark red crystalline mass was obtained which on further crystallisation from nitrobenzene appeared as light red glistening crystals. These were washed with alcohol and ether. Both the pyruvic acid hydrazone and the methylglyoxal osazone were dried for 24 hours in a vacuum desiccator.

Previously to this, specimens of the hydrazone and osazone had been prepared from actual pyruvic acid and from a solution of methylglyoxal (made according to Neuberg and Hofmann [1930]), and had been purified in the same way.

Melting-points:

(*a*) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from pyruvic acid) Neuberg and Kobel [1929] give 216°, Allen [1930] gives 213°	214°	(uncorr.)
(*b*) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from muscle) ...	214°	,,
(*c*) Mixed melting-point	214–215°	,,
(*d*) Methylglyoxal 2 : 4-dinitrophenylosazone (from methylglyoxal) Neuberg and Kobel [1928] give 298°	297·5°	,,
(*e*) Methylglyoxal 2 : 4-dinitrophenylosazone (from muscle) ...	297°	,,
(*f*) Mixed melting-point	296°	,,

From a study of the foregoing Tables, notably III and VI, it will be observed that pyruvate, as demonstrated by the nitroprusside test, is sometimes to be met with in the solutions during the very early periods of the incubation. Where sulphite is present the quantity of pyruvate tends to increase as the incubation proceeds, but in the absence of sulphite the pyruvate progressively disappears. If this compound is indeed a transitory phase of lactic acid production, it is to be expected that immediately after a treatment such as mincing, which is strongly provocative of rapid glycolysis, the system will contain relatively large quantities of pyruvic acid. It is also apparent from the Tables that in the absence of a fixative this pyruvate very quickly disappears. It was therefore deemed of interest to try the effect of the addition of pyruvate to minced muscle both in presence and absence of sulphite, and the following experiment was accordingly performed.

A. 120 g. minced muscle
25 cc. buffer (× 10)
5 cc. 1 % potassium pyruvate
Water to 250 cc.

B. 120 g. minced muscle
25 cc. buffer (× 10)
5 cc. 1 % potassium pyruvate
6·5 cc. *M* Na_2SO_3
Water to 250 cc.

The experiment was aerobic. Nitroprusside tests were carried out as usual at intervals, the first test being made as soon as possible after setting up the mixtures. This time an attempt was made to render the tests to some extent quantitative.

For each test 5 cc. of the muslin filtrate were precipitated with 1 cc. of 50 % trichloroacetic acid. After filtering, 1 cc. of the clear solution was taken for estimation. The reagents employed were:

(*a*) 40 % acetic acid;

(*b*) freshly prepared 1 % aqueous sodium nitroprusside;

(*c*) ammonia (sp. gr. 0·880) diluted with an equal volume of water and saturated with ammonium sulphate.

The 1 cc. of solution to be tested was mixed with 0·5 cc. of (*a*), 3·0 cc. of (*b*) and 1·5 cc. of (*c*) in the order named.

Simultaneously, standards were set up consisting of 1 cc. of various dilutions of potassium pyruvate. These standards were prepared by mixing 5 cc. of a solution containing 2 mg. pyruvic acid in 5 cc. with 1 cc. of 50 % trichloroacetic acid, taking 1 cc. of this final mixture and adding the reagents as described above. The standard designated as I thus contained 0·34 mg. pyruvic acid in 1 cc. Standard II was half of this concentration, while standard III was one-quarter, and standard IV one-eighth.

The strength of the blue colours obtained in the unknowns was compared visually with the standards, a colorimeter not being employed.

Table VIII.

Time after commencement of exp. (mins.)	A	B
2	Negative (<IV)	Midway between I and II
30	Negative (<IV)	Slightly >I
90	Negative (<IV)	>I
240	Negative (<IV)	Considerably >I

Since 250 cc. of the original fluid contained 50 mg. pyruvic acid, the 1 cc. taken for testing would contain 0·17 mg. of pyruvic acid originally added. The above Table, approximate only though it is, shows therefore that (*a*) within a few minutes of adding the muscle there is a considerable increase in pyruvic acid in the case of the experiment with sulphite, and the value rises ultimately to more than twice the original figure, and (*b*) in the experiment without sulphite, all the added pyruvate has disappeared from the solution within 2 minutes.

It is hoped in future work to make this test more exactly quantitative, and experimentation is being carried out with this end in view.

As an additional check upon the information afforded by the above figures, the residual fluid in B (190 cc.) was treated with 2 : 4-dinitrophenylhydrazine in the usual way and after extraction with 25 % sodium carbonate and reprecipitation with HCl, to free it from the methylglyoxal derivative, was weighed. At the same time 190 cc. of a solution of the same composition as B, to which no muscle had been added, was similarly treated and the precipitate weighed.

Weight of 2 : 4-dinitrophenylhydrazone from B	0·222 g.
,, ,, ,, from solution of same composition	0·107 g.

This furnishes confirmation of Table VIII.

Experiments on frog muscle.

Although at the inception of this work frog muscles were exclusively used, and rabbit muscle was not employed till later on, it has been found more

convenient for the purposes of this paper to describe these latter experiments before those which are about to be reported. The methods used for detecting and identifying pyruvic acid and methylglyoxal are identical in the two series, and hence need not be further described in detail.

In all the experiments reported below, when muscles are referred to as "stimulated," the technique consisted in immersing the limbs in the appropriate solutions in small beakers, and, by means of a small induction coil, stimulating indirectly *via* the nerves. It has since been found more effective to apply direct stimulation, as in the presence of compounds such as sulphite the nerve seems to lose its irritability much sooner than does the muscle. Those portions of wire actually in contact with the muscle were always of silver. Where muscle is described as "chopped" it was dissected from the bone and chopped fairly finely with scissors. In aerobic experiments oxygen was bubbled through the solutions bathing the muscles, while in anaerobic ones the beakers containing the muscles were enclosed in a large vacuum desiccator on the bottom of which was a layer of alkaline pyrogallol, while the desiccator itself was filled with nitrogen by repeated evacuation and replenishment from a nitrogen cylinder. The wires for stimulation in such cases were led out through the rubber stopper.

It will be best, for the sake of brevity, to present the whole series of experiments in a consecutive manner and then discuss the significance of the results.

Exp. I.

A. 20 cc. frog Ringer solution (× 10)
20 cc. phosphate buffer, p_H 7·2 (× 10)
Water to 200 cc.

B. As A, with the addition of 5 cc. *M* Na_2SO_3

Two limb-pairs, severed at the girdle, were placed in each and were stimulated as long as irritability lasted. The experiment was aerobic and continued for 5 hours. The muscles were then ground with sand and water (15 cc.). Nitroprusside tests were done on the muslin filtrates.

A	B
–	+ + + +

Exp. II.

A. 5 cc. frog Ringer solution (× 10)
5 cc. buffer (× 10)
5 cc. *M*/10 sodium lactate
Water to 100 cc.

B. As A, with the addition of 3 cc. *M* Na_2SO_3

In each beaker were placed the muscles from three limb-pairs, dissected carefully from the bones, but not chopped. Oxygen was bubbled for 4 hours, after which the muscles were ground with sand and trichloroacetic acid.

The nitroprusside reactions were negative for pyruvate in both cases.

The *p*-nitrophenylhydrazine derivatives were prepared; B yielded about twice as much as A. After washing, these both gave a strong pure blue colour

with alcoholic potassium hydroxide, indicating that the product was in each case methylglyoxal *p*-nitrophenylosazone.

Exp. III.

A and B. 10 cc. buffer (× 10)
10 cc. frog Ringer solution (× 10)
5 cc. *M*/10 sodium lactate
2·5 cc. *M* Na_2SO_3
Water to 100 cc.

One limb-pair was placed in each, the only difference being that the mucles in A were stimulated, while those in B were not. The experiment was aerobic and lasted for 5 hours. The muscles were then ground with sand and 15 cc. of their respective fluids.

Nitroprusside reactions:

A	B
+ + + +	+

The remainder of the filtrates were deproteinised with trichloroacetic acid, filtered, concentrated, and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep red. Indicative of pyruvic acid	"Mixed" colour, with preponderance of blue. Indicative of methylglyoxal, slightly contaminated with pyruvic acid

Exp. IV.

A.	B.
10 cc. buffer (× 10)	10 cc. frog Ringer solution (× 10)
3 cc. *M* Na_2SO_3	3 cc. *M* Na_2SO_3
Water to 100 cc.	Water to 100 cc.
Chopped muscle from one limb-pair	One intact limb-pair, not stimulated

The experiment was aerobic and lasted for 6 hours. Both lots of muscle were then ground with sand and water.

Nitroprusside reactions:

A	B
−	+ + + +

The remainder of the filtrates were deproteinised and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep blue. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid

Exp. V.

50 cc. frog Ringer solution (× 10)
12·5 cc. *M* Na_2SO_3
0·25 g. $NaHCO_3$
Water to 500 cc.

A. Two limb-pairs unstimulated in 100 cc.	Aerobic
B. Two limb-pairs stimulated in 100 cc.	Aerobic
C. Muscle removed from 4 legs (not chopped) in 50 cc.	Aerobic
D. Two limb-pairs unstimulated in 100 cc.	Anaerobic
E. Two limb-pairs stimulated in 100 cc.	Anaerobic
F. Muscle removed from 3 legs (not chopped) in 50 cc.	Anaerobic

The limbs were chosen so that the weight of muscle in each vessel was approximately the same (about 8 g.). The duration of the experiment was 2 hours; the muscles were then ground with sand and 15 cc. of water.

Nitroprusside reactions:

A	B	C	D	E	F
+	+ + +	+	+ +	+ + + + +	+

Iodine titrations for bound sulphite (on 2 cc. of filtrate from ground tissue, and 5 cc. of fluids in vessels). Strength of iodine 0·008 *N*.

	A	B	C	D	E	F
Fluid	0·10	0·15	0·91	0·15	0·20	0·55
Tissue	3·60	3·60	2·80	4·30	4·60	2·50

These iodine figures bear out the results of the nitroprusside tests in that in D and E (the anaerobic whole limbs) the bound sulphite is greater in amount than in the corresponding aerobic limbs A and B.

They show also that in the case of intact muscles diffusion of the metabolic products into the surrounding fluid is almost negligible.

The main facts emerging from this series of experiments, which is of necessity as yet far from complete, are as follows.

(i) Stimulation always leads to an increased amount of pyruvic acid in the tissue (Exps. I, III, V).

(ii) Pyruvic acid can be demonstrated to occur even in the absence of sulphite (Exp. I).

(iii) Aerobic conditions are not essential for the production of pyruvic acid, which indeed on the other hand appears to be favoured by anaerobiosis (Exp. V).

(iv) As with rabbit muscle, methylglyoxal can also be detected (Exps. II, III, IV), and in presence of sulphite seems to be fixed to a greater extent (Exp. II). This compound appears to be produced under conditions which are not yet determined, but it is noteworthy that in cases where the muscle has been removed from the limbs, as in Exp. II, methylglyoxal always seems to occur rather than pyruvic acid. This has frequently been found to be the case in other similar experiments not reported here, but at present we have no idea of what the significance of this circumstance may be.

At all events, on the whole the experiments with frog muscle confirm strikingly the results obtained with rabbit muscle. The favourable influence of stimulation upon pyruvic acid formation is evidence for the intermediary occurrence of this substance during lactic acid production, as is also the absence of any sign of inhibition of its formation under anaerobic conditions.

Discussion.

The results recorded in this paper are to be regarded as being of a preliminary nature only. Much more work will have to be accomplished before

the great technical difficulties associated with a problem of this kind can be surmounted to such an extent as to render the results quantitative.

We believe that we have fairly conclusively shown that both pyruvic acid and methylglyoxal do participate at any rate in some process or other occurring in muscle. With regard to the factors conditioning their appearance or non-appearance there is practically nothing that can at present be said with any degree of certainty. There can be no doubt that these substances in their relation to one another constitute part of a very delicately balanced system, the equilibrium of which is likely to be disturbed by seemingly slight variations in the surrounding medium and conditions. This relation, it is intended, will form the subject of further research.

That pyruvic acid is of importance in the metabolism of muscle is strongly suggested by the recent work of Toeniessen and Brinkmann [1930], who demonstrated a rapid removal of this substance when perfused through the musculature of a rabbit. Our own results with minced muscle confirm this rapid disappearance.

It has long been a widely-held view that methylglyoxal occupies an important position in the series of changes resulting in lactic acid production in muscle, and our results are in accord with this also.

Very many fresh experiments suggest themselves, which we propose to make the subject-matter of a future communication. Among these may be mentioned a study of pyruvic acid formation in muscle poisoned with iodo-acetic acid, with fluoride and with amylase. The bearing of such experiments upon the question as to whether pyruvic acid and methylglyoxal are indeed being formed during the change carbohydrate $\rightarrow$lactic acid is obvious.

The possibility must not be overlooked that lactic acid may be oxidised to pyruvic acid anaerobically in the presence of hydrogen acceptors in the muscle, or even that the source of the pyruvic acid may not be carbohydrate in nature at all, but that for instance this compound may be an intermediary in fat metabolism. On the whole, however, we are not inclined to regard either of these hypotheses as very probable.

Summary.

1. The technique of the use of sodium sulphite as a fixative has been employed for investigating some of the substances concerned in the intermediary metabolism of muscle.

2. Pyruvic acid and methylglyoxal have been shown to occur in frog and rabbit muscle, both in the presence and absence of sulphite, and under both aerobic and anaerobic conditions. These substances have been isolated and identified as the 2 : 4-dinitrophenyl-hydrazone and -osazone respectively.

3. In view of the conditions, in so far as they have been established, under which these compounds make their appearance, it is suggested that they may both be intermediary between carbohydrate and lactic acid.

It is with much pleasure that we express our thanks to Sir F. G. Hopkins and Prof. J. B. S. Haldane for their interest in this work.

REFERENCES.

Allen (1930). *J. Amer. Chem. Soc.* **52**, 2955.
Case (1929). *Biochem. J.* **23**, 210.
Cook (1930). *Biochem. J.* **24**, 1526.
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 127.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Hahn, Fischbach and Haarman (1928). *Z. Biol.* **88**, 89.
Neuberg and Gottschalk (1925). *Biochem. Z.* **158**, 253.
—— and Hofmann (1930). *Biochem. Z.* **224**, 491.
—— and Kobel (1928). *Biochem. Z.* **203**, 463.
—— —— (1929). *Biochem. Z.* **216**, 493.
—— —— (1930). *Ber. deutsch. chem. Ges.* **63** B, 1986.
—— and Reinfurth (1920). *Ber. deutsch. chem. Ges.* **53** B, 1039.
Rimini (1904). *Z. anal. Chem.* **43**, 517.
Simon and Piaux (1924). *Bull. Soc. Chim. Biol.* **6**, 477.
Toeniessen and Brinkmann (1930). *Z. physiol. Chem.* **187**, 137.

CXLV. PIGMENTATION OF THE LIVER OF THE MONK (OR ANGLER) FISH.

By JOHN ARNOLD LOVERN and RICHARD ALAN MORTON.

From the Torry Research Station, Aberdeen, and The University, Liverpool.

(*Received July 3rd, 1931.*)

In studying the livers of various species of fish, one of us (J. A. L.) noticed the frequent occurrence of a pronounced pink pigmentation in the interior portions of the liver of the monk fish (*Lophius piscatorius*). The livers of gadoid fish, such as codling, hake, *etc.*, are of uniform colour throughout, being in most cases almost white. Colouring is sometimes seen in the Elasmobranchii (*e.g. Squalus acanthias* and *Raia clavata*) but as a rule the livers are very pale. The exceptions are mottled in appearance, and the pigmentation is due to a grey-black substance, which is not extracted along with the oil.

The pigmentation of monk-livers is, therefore, a distinct and unusual phenomenon. The colouring matter is localised in irregularly shaped deposits found much more often in the interior than in the exterior portions of the liver. The intensity of the pigmentation varies between wide limits, not only from fish to fish, but also for the localised deposits in a particular liver. In an absolutely fresh liver the intensity of the pigment is much less than in the same liver after exposure to air, and oxidation thus appears to play a part in its formation.

Histological examination of the pigmented areas shows that not all the cells are pigmented, even in such areas. The distribution of pigment appears to be quite irregular. Under high power the pigment is seen to be present in the form of deep orange droplets (possibly dissolved in oil), of various sizes, but of the same order of magnitude as the liver cells. It is worth noting that the formaldehyde fixative used did not appreciably bleach the pigment. Bleaching experiments with a strong solution of ferric chloride in 50 % alcohol, in which pigmented histological sections were immersed, revealed a fairly rapid and complete bleaching. Two examples may be given.

Section from liver No. 1.

Pigmented section after 5 minutes in $FeCl_3$	No appreciable bleaching
Same section after 20 minutes in $FeCl_3$	Complete bleaching of pigmented areas

Section from liver No. 2.

Pigmented section after 5 minutes in $FeCl_3$	Some bleaching
Same section after 20 minutes in $FeCl_3$	Mainly bleached
Same section after 30 minutes in $FeCl_3$	Completely bleached

The pigment accompanies the oil when the livers are extracted by means of solvents. It is noteworthy that oil cannot be obtained from these livers by steaming, although the oil content is frequently as much as 50 %. In one experiment a small portion of the unsaponifiable matter was prepared and found to contain much of the pigment present in the original oil. This unsaponifiable matter (mainly cholesterol) was spread in a thin film on a glass slip and exposed to air and sunlight for some days. Bleaching was only gradual, over a week elapsing before the colour had all disappeared.

The nature of this pigment has not yet been fully elucidated, and the investigation is being continued in the hope of isolating a quantity sufficient for identification. Some interesting properties have, however, already emerged. Previously to commencing the study of monk-livers it had been found that oils prepared from the various parts of any particular fish-liver gave the same intensity of blue colour with antimony trichloride. In the case of the first three monk-livers examined, however, the following results were obtained (J. A. L.).

		Blue value (Carr-Price)
Liver (1)	Oil from pigmented interior	9·6
	Oil from non-pigmented exterior	3·6
Liver (2)	Oil from pigmented interior	4·4
	Oil from non-pigmented exterior	2·4
Liver (3)	Oil from pigmented interior	14·0
	Oil from non-pigmented exterior	3·2

In view of the well-known chromogenic properties of carotenoids it appeared worth while to investigate whether the pigment was carotenoid in type, especially since in the case of a monk-liver which apparently contained no pigment at all, the oils from the inside and outside layers gave the same intensity of blue colour. However, in later experiments it was found that this difference between the chromogenic power of the oils from the pigmented and non-pigmented parts is not always observed. Thus, in the case of a liver having a very intense localised pigmentation, the oils from the red and white parts of the liver respectively gave the same blue colour in the antimony trichloride test, namely 2·8 Carr-Price units. The intensities of the blue colours were thus not proportional to the redness of the oils.

It was decided to examine the oils spectroscopically in order to see whether additional evidence as to the nature of the coloured substance could be obtained. The following samples were studied.

Sample (A). The mixed oils from livers (1), (2) and (3). (These were unfortunately mixed for other purposes before experiments on the nature of the pigment were undertaken.)

Sample (A'). The unsaponifiable matter from A, dissolved in chloroform.

Sample (B). Oil from the inside of a monk-liver.

Sample (C). Oil from the outside of the same liver exhibiting little or no pigmentation.

Sample (D). Oil from non-pigmented parts of a liver with deeply pigmented deposits.

Sample (E). Oil from pigmented parts of the same liver.

Sample (F). Ether-extracted oil from several complete livers exhibiting a fairly uniform distribution of a rather low intensity of pigmentation.

All the oils showed the band at 328 $m\mu$ as a faint inflexion, superimposed on much more intense general absorption.

Samples (A), (E) and (F) gave bands with maxima at 480–485 $m\mu$, 450–457 $m\mu$ and 422–430 $m\mu$, in the same region and of the same type as those given by carotenoid pigments such as carotene or xanthophyll. At the same time the maxima do not agree exactly with those of carotene dissolved in an oily medium (*e.g.* arachis oil). Identification of definite members of the carotenoid group is a difficult matter, especially in the presence of vitamin A,

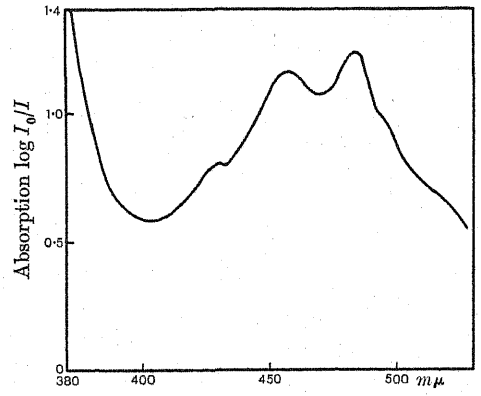


Fig. 1. Monk-liver oil F_1. $\frac{1}{2}$ mm. layer.

so that although the evidence suggests a carotenoid, it is by no means conclusive. It may be significant that materials containing the pigment (both in oils and extracts of the unsaponifiable matter dissolved in ether or chloroform) respond to many of the colour tests for carotenoids, *e.g.* with strong sulphuric acid there is a gradual development of an intense green colour, which, although gradually fading, persists for 24 hours. There is some doubt as to the product of the interaction of the pigment and antimony trichloride. The blue solution obtained with carotene exhibits a maximum near 590 $m\mu$, and this could not be detected, whilst the blue colour with monk-liver oil exhibited the two bands with maxima at 572 $m\mu$ and 606 $m\mu$ respectively associated with vitamin A. The presence of these maxima may mask absorption in the region of the 592 $m\mu$ band and render detection difficult.

It would thus appear that whilst some of the evidence suggests a carotenoid nature for the pigment, there is no satisfactory evidence as to any particular carotenoid—as stated above it is hoped to isolate the pure pigment in sufficient quantity for identification. If it should prove to be carotenoid in nature, it

may well provide additional evidence in the carotene-vitamin A problem. It is interesting to note that the vitamin A content of the livers of *Lophius piscatorius* is generally low compared with other species.

Experiments on monk-liver oils.

Oil	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ (gross)	Bands in the visible spectrum	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
A	1·4	480, 450, 422 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	1·0	0·55
Unsaponifiable matter	—	482, 452, 420, 400 $m\mu$	—	—
B	0·26	Ill-defined	0·24	0·2
C	—	Ill-defined	0·2	0·2
D	0·7	Inflexion only	0·42	0·12
E	0·85	485, 455, 422 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	0·44	0·24
F	0·82	485, 455, 430 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	0·825	0·25

Discussion.

The spectroscopic data on the monk-liver oils indicate that the vitamin A potency varies between 0·2 and 0·8 of that associated with an average cod-liver oil (1·0 spectroscopic unit or about 12–13 Carr-Price units). The particular samples studied all showed a clear maximum at 572 $m\mu$ in the antimony trichloride colour test, but the capacity to give the more usual 606 $m\mu$ maximum was latent [*cf.* Heilbron, Gillam and Morton, 1931]. The ultra-violet absorption band at 328 $m\mu$, characteristic of vitamin A appears to be present in the intensity to be expected from the colour test in all the oils, but owing to superimposed, more general absorption, the band only appears as a marked inflexion.

There appears to be no direct connection between the pigment and vitamin A, as the non-pigmented oils cannot be differentiated from the pigmented oils by any property other than colour.

The pigment resembles carotene in its absorption in the visible spectrum. Since 0·5 mm. layers of different oils exhibit the three bands at 480, 450 and 422 $m\mu$, the points of maximum absorption corresponding with

$$E = \log I_0/I = 0{\cdot}8 - 1{\cdot}2,$$

we have

$$E^{1\%}_{1\,cm.}\ 450\,m\mu = 0{\cdot}16 - 0{\cdot}24.$$

Now for carotene itself $E^{1\%}_{1\,cm.}$ 450–460 $m\mu$ = 1400, whilst the intensity of the blue solution obtained with carotene and antimony trichloride is of the order $E^{1\%}_{1\,cm.}$ 590 $m\mu$ = 300–400. Accepting the higher figure, we can calculate approximately the intensity of a blue colour due to the quantity of pigment present in monk-liver oil on the assumption that it is carotene. The value for $E^{1\%}_{1\,cm.}$ 590 $m\mu$ is 0·04 to 0·07. Now in the present series of monk-liver oils the values for $E^{1\%}_{1\,cm.}$ 572 and $E^{1\%}_{1\,cm.}$ 606 $m\mu$ are never less than 0·20. Hence we have

the rather important conclusion that, deeply pigmented as the monk-oils are, the quantity of vitamin A present even in oils having only one-fifth of the potency of cod-liver oil, is sufficient to give a blue colour from 3 to 6 times as intense as that to be expected from the pigment on the basis that it is a carotenoid. It is thus not very surprising that the pigment did not give rise to a distinct detectable band in the colour test.

There is now considerable evidence that carotene is heterogeneous, containing two or more closely related substances. From the present point of view this fact increases the difficulties in identifying the pigment of monk-livers. We are endeavouring to secure pigmented monk-liver oils which are either very poor in vitamin A or relatively very rich in pigment, but, as the conditions governing seasonal and other variations in potency and pigmentation are unknown, the test may well prove difficult.

SUMMARY.

Monk-livers often contain irregular deposits of a red fat-soluble pigment, hitherto not found in fish-livers. The pigment appears to act as a chromogen towards antimony trichloride, but quite small quantities of vitamin A are so intensely chromogenic as to obscure the rôle of the pigment. Such evidence as is available points to the pigment being carotenoid in type.

We are indebted to the Department for Scientific and Industrial Research for support granted to this investigation.

REFERENCE.

Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.

CXLVI. THE MITTELMANN PROCESS FOR TREATING FISH-LIVERS.

By JOHN ARNOLD LOVERN, RICHARD HENRY CREED and RICHARD ALAN MORTON.

From the Torry Research Station, Aberdeen, and The University, Liverpool.

(*Received July 3rd, 1931.*)

Two reports by Mittelmann on "Les graisses de la Morue et nouvelles méthodes de leur préparation" [1925] and "The manufacturing of fish conserves of Murman" [1927] are somewhat difficult of access, and this may account for the fact that certain distinctly important claims have so far neither been confirmed nor refuted in the literature. We are indebted to Bezssonoff [1929] for a summary of Mittelmann's work, the gist of which is as follows. Fresh cod-livers are sealed in evacuated tins which are then heated to 120°, the process amounting to autoclaving the livers. On opening the tins the liberated oil can readily be poured off, and it is said to be pleasant in taste and free from the typical disagreeable smell characteristic of all cod-liver oils prepared by any method used in the industry. "On testing the oil immediately after opening a tin no blue colour with antimony trichloride was obtained, but if the oil was re-examined after 10 days a positive colour test was recorded. At the same time, the characteristic unpleasant smell of commercial cod-liver oils began to manifest itself. Thus, contact with air causes the appearance of the evil-smelling substance (probably as a result of the formation of bodies with ethenoid linkages) and simultaneously brings into being the property of giving a blue colour with antimony trichloride."

The importance of these claims is considerable both from the theoretical and practical standpoints. If vitamin A is really present in the oils from freshly opened tins the antimony trichloride colour test must have serious limitations, to say the least. If, on the other hand, the colour test is definitely a valid measure of vitamin A, then since negative results are obtained with freshly opened tins, the oil from the autoclaving process must be devoid of vitamin A. As, however, the chromogenic property of the oil is acquired on standing in air, the vitamin must be an oxidation product of a precursor substance stored in the liver.

These implications made it necessary in our opinion to undertake a careful repetition of the work of Mittelmann. Accordingly arrangements were made for the livers of fish caught by Aberdeen trawlers to be canned and autoclaved at the Torry Research Station and the oils examined by the Lovibond technique applied to the antimony trichloride colour test. The tins were also to

be sent to Liverpool with the minimum delay so that a complete spectroscopic examination could be carried out. We are indebted to Mr John Bruce of Messrs Marshall and Co., Ltd., of Aberdeen for assistance in canning the livers. The tins used were lacquered on the inside to guard against contamination by the metal, the lacquer being chemically inert and oil-resisting. Tins of one pound capacity were used as these were the largest which could be sealed under vacuum. They were not large enough to contain whole livers, so that in all cases except those of small codling, the livers were cut into pieces before packing. After evacuating to 1 inch of mercury and sealing, the tins were autoclaved at 120° for 2 hours.

The first experiments were carried out on small codling caught off Aberdeen, and the livers were only about 4 hours old when canned. The oil obtained from the tins was examined the same day (19. ii. 1931) and gave a reading of 1·84 Carr-Price blue units (Lovibond). After storage under nitrogen for 6 days the value was 2·24 units, and after 13 days 2·28 units.

Ultra-violet absorption spectra for the oil from freshly opened tins, photographed less than 3 days after the fish were caught, showed that the codling-liver oils were remarkably transparent. With a 1 mm. layer of the oil itself (no solvent) 3 % of the incident light was transmitted at 299·5 $m\mu$, the curve showing a small maximum at 320–330 $m\mu$, the value for the gross uncorrected absorption at this point being E (= $\log I_0/I$) 0·125 reduced to the basis of a 1 % solution and a 1 cm. cell. With antimony trichloride a definite blue-violet colour was produced, the solution exhibiting a well-defined maximum at 572 $m\mu$. With most cod-liver oils the blue solution is characterised by a maximum at 606 $m\mu$, the spectral absorption curve being unsymmetrical owing to the presence of greater or smaller absorption at 572 $m\mu$, which is usually less intense than that at 606 $m\mu$. With the codling-liver oil from freshly opened tins, there was definite, measurable absorption at 606 $m\mu$, but this was approximately only half as intense as the absorption at 572 $m\mu$. On standing exposed to air, or on oxidation with oxygen at 70°, the capacity to give a blue colour with antimony trichloride underwent a considerable increase. This was reflected in the spectral absorption of the blue solution by an increase in absorption at 606 $m\mu$, which was not accompanied by any appreciable increase or decrease at 572 $m\mu$.

A number of larger codling-livers were also canned and autoclaved at Aberdeen and examined spectroscopically at Liverpool. Here again, the freshly opened tins yielded oils which exhibited a small absorption band at 328 $m\mu$ and gave a colour test in which the 572 $m\mu$ band predominated over the 606 $m\mu$ maximum. It is interesting to note that the blue solution with antimony trichloride slowly faded to a clear yellow colour characterised by a sharp maximum at 460–465 $m\mu$.

The preliminary experiments on codling-livers having given indications of an increase in the chromogenic value of the oils from autoclaved livers on standing, it was decided to repeat the experiments under more defined con-

ditions and with adequate controls. Accordingly ½ cwt. of cod-livers were obtained from fish landed at Buckie (3. iii. 1931) and gutted on shore. Nets had been out since 28. ii. 1931 so that many of the fish had been dead for a few days. The livers were conveyed to Aberdeen and each was cut into two roughly equal parts. One half of the catch was canned and autoclaved at 120°, whilst the other half was steamed with direct steam in the usual way for preparing cod-liver oil.

The Buckie cod were on spawn at the time of catching, whereas the codling already referred to were either immature or just about to spawn. It must be emphasised that the steamed liver oil was obtained from half the whole catch, whereas individual tins subjected to the Mittelmann process would be representative of two or three livers, so that the experiment could only afford a qualitative control, the well-known variations in potency of the oil from individual livers precluding strictly quantitative control.

Lovibond readings obtained at Torry Research Station on March 4th, 1931, gave readings of 4·0 and 6·4 Carr-Price units for the oils from autoclaved and steamed livers respectively. After 3 days' storage under nitrogen the oils gave values of 8·0 and 7·2 blue units. The oils and tins reached Liverpool on March 5th, and were examined on that and the following days. The spectroscopic examination of the antimony trichloride-oil solution gave results indicating that little or no change had occurred in transit. The blue solution showed a well-defined maximum at 572 $m\mu$ together with marked, but much less intense, absorption at 606 $m\mu$. The ultra-violet band with maximum near 328 $m\mu$ was shown very clearly, but at an intensity much greater than would be expected from the proportion of the chromogen responsible for the 606 $m\mu$ absorption in the colour test.

Different tins were opened at various times for several weeks after receiving the oils. It was found that in the sealed tins the oil gradually underwent a change which resulted in enhanced absorption at 606 $m\mu$, but within the limits of error no appreciable change occurred either in the absorption at 572 $m\mu$ or 328 $m\mu$ in the ultra-violet spectrum. It was at any rate clear that the marked predominance of the 572 $m\mu$ band slowly disappeared. Storage of the oil in bottles under nitrogen likewise failed to prevent a slow change resulting finally in a very marked increase in the 606 $m\mu$ band relatively to the 572 $m\mu$ band. Oxidation with oxygen at 70° or ozone at room temperatures likewise caused a marked increase in the 606 $m\mu$ band, which in this case was ascertainably not at the expense of a diminution either of the 572 $m\mu$ band or the ultra-violet band. It is noteworthy that the blue solution again faded to a bright yellow marked by a maximum at 465 $m\mu$.

A few comments on Mittelmann's process as a method for the manufacture of cod-liver oil are necessary. In our hands, the yield of oil is poor and variable, in some cases more water than oil being obtained. In nearly every case it was found necessary to dry the oil with anhydrous sodium sulphate before carrying out any tests. It is moreover very difficult to free the liver débris

from oil. Comparing the autoclaved liver oil with the steamed liver oil prepared from the same livers, there is no doubt that the steaming process can, at any rate in the laboratory, give oils superior in taste, odour and colour. The oil from the tins has a peculiar "meaty" taste and smell, quite different from, and perhaps worse than, the fishy taste of medicinal cod-liver oil. If the oils are kept for some weeks in bottles, or if the tins are not opened for some weeks, there is a very noticeable improvement in taste and smell. As regards colour, the oils are very pale, but not quite so good in this respect as the steamed oil obtained under laboratory conditions. The oil from the tins exhibits less tendency to deposit stearin than the steamed oils, and this is probably a consequence of the prolonged heating of the oil, as crude cod-liver oil deposits much less stearin after heating than before.

Drummond and Hilditch [1930] have expressed the view that vitamin A is not necessarily associated with the fat in the liver, the fat merely dissolving the vitamin on disruption of the cells. It may therefore be significant that the liver tissue does not appear to be so completely disintegrated by Mittelmann's process as by careful and adequate steaming.

Summary of data.

Oil	$E^{1\%}_{1\,cm.}$ 606 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 328 $m\mu$
Small codling-livers (autoclaved):			
(*a*) Freshly opened tins	0·06	0·12	—
(*b*) After 4 hours	0·06	0·12	0·125
(*c*) After 24 hours	—	—	0·16
Ditto, treated with oxygen for 5 mins. at 70°:			
(*a*) Examined at once	0·10	0·12	—
(*b*) Left overnight	0·18	0·12	—
Larger codling-livers:			
Freshly opened tins	0·13	0·20	—
,, 4 hours later	0·13	0·20	0·22
Two more tins (freshly opened)	0·125	0·175	—
Oxidised	0·32	0·175	0·21
Steamed cod-livers: Fresh	0·35	0·76	0·73
Autoclaved cod-livers:			
Freshly opened tins	0·27	0·60	—
Two more freshly opened tins	0·30	0·63	—
Steamed cod-livers: 12 days old	0·60	0·78	0·75
Autoclaved cod-livers:			
10 tins opened 10 days after canning	0·39	0·72	0·70
9 days after opening	0·42	0·67	—
3 tins opened 3 weeks after canning	0·61	0·94	—
5 tins opened 3 months after canning	0·36	0·54	—

Discussion.

The most important result emerging from the work is the confirmation of Mittelmann's observation that the chromogenic value of fresh cod-liver oils sometimes increases on standing for a relatively short time. We have not so far encountered a cod-liver oil which failed to respond qualitatively to the colour test, but with codling-, cod- and monk-liver oils it is clear that the chromogenic values may increase on oxidation. Inasmuch as the experiments

with the steamed cod-liver oil indicated precisely the same tendency towards an increase in the blue value as the autoclaved oils, there is no evidence for regarding the Mittelmann process *per se* as having any special significance. Moreover, the ultra-violet band, which is at present perhaps the least debatable criterion of vitamin A, discloses no qualitative differences between autoclaved liver oils and oils prepared by other processes. The results agree well with the conception advanced in the following papers, that liver oils contain two distinct if not unrelated chromogens.

We are indebted to the Committee on Research of the University of Liverpool and to the Department for Scientific and Industrial Research for financial assistance towards the cost of this investigation.

REFERENCES.

Bezssonoff (1929). *Bull. Soc. Chim. Biol*, **11**, 1146.
Mittelmann (1925). *Nouvelles Inst. Agronom. Exp.* **3**, 1.
—— (1927). *Trans. Inst. for Explor. of the North, Leningrad*, 38, 39.
Drummond and Hilditch (1930). *Empire Marketing Board, Report* 35.

CXLVII. THE ANTIMONY TRICHLORIDE COLOUR TEST AND THE ULTRA-VIOLET ABSORPTION OF LIVER OILS AND CONCENTRATES.

BY ALBERT EDWARD GILLAM AND RICHARD ALAN MORTON.

From the Department of Chemistry, The University of Liverpool.

(*Received July 3rd, 1931.*)

IN a recent paper [Morton, Heilbron and Thompson, 1931] it was stated that the blue solution obtained by the interaction of liver oils and antimony trichloride was liable to exhibit an absorption maximum at 572 $m\mu$, instead of, or in addition to, the maximum at 606 $m\mu$ which has hitherto been studied [Morton and Heilbron, 1928; Drummond and Morton, 1929]. It was also recorded that with concentrates a maximum at 583 $m\mu$ occasionally made its appearance with or without the simultaneous appearance of the more usual band at 620 $m\mu$. No wholly satisfactory explanation of these observations being forthcoming, it was decided to examine a large number of oils and concentrates as completely as possible with a view to re-testing the correlation between ultra-violet absorption [Morton and Heilbron, 1928] and the spectroscopic data on the colour test. For this purpose, the intensities of absorption at 572 $m\mu$ and 606 $m\mu$ were determined for the blue solutions, irrespective of whether one or two maxima were visually apparent in the eye-piece of the spectroscope.

From the results of Drummond and Morton [1929] it is clear that the blue colour with antimony trichloride and the ultra-violet band at 328 $m\mu$ both vary in intensity exactly as would be expected if both were due to vitamin A. The experimental error of the biological assay is however so great that although the correlation of these properties with the vitamin is highly probable, it is not proved in a rigid quantitative sense.

The ultra-violet band is however on a different footing from the blue colour. It provides a mechanism for the photochemical destruction of vitamin A and itself disappears on irradiation. The band moreover appears in the liver-fat of rats fed on a vitamin A-deficient diet with carotene as a growth-promoting supplement, such fat being itself potent in vitamin A. The band is however absent from the liver-fat of rats maintained on a vitamin A-free diet without added carotene [Moore, 1930; Drummond, Ahmad and Morton, 1930]. Similar considerations indicate that absence of chromogen coincides with absence of vitamin, and presence of chromogen coincides with presence of vitamin. This state of affairs would be not unsatisfactory were it evident that the chromogenic property of liver oils is due to a single, well-defined entity. The colour-

producing mechanism is however complicated and difficult to describe unambiguously. The displacement from 606 to 620 $m\mu$ shown by concentrates is troublesome in the sense that it lessens the precision of the description of the blue colour. It raises the question as to whether purer preparations of vitamin A would give rise to an enhanced displacement. Moreover the appearance of bands at 572 or 583 $m\mu$ in certain oils and concentrates can only mean an unusual variability of the colour substance or complex, or else the existence of a new chromogen in certain oils and concentrates. If a new chromogen can appear in a few oils, the possibility cannot be evaded that all oils may contain varying proportions of two chromogens.

As a first step in investigating this matter, attention may be drawn to the fact that if a series of oils is tabulated in order of increasing intensity of absorption at 328 $m\mu$, the absorption at 606 $m\mu$ should increase steadily down the table. Examination of Table I, which shows the data for 52 liver oils in

Table I. *Cod- and other fish-liver oils in which the value of* $E^{1\%}_{1\,cm.}$ *328* mμ *is less than the value of* $E^{1\%}_{1\,cm.}$ *606* mμ.

328 $m\mu$	606 $m\mu$	328 $m\mu$	606 $m\mu$
0·14	0·15	1·38	1·59
0·5	0·54	1·40	1·60
0·5	0·84	1·50	1·51
0·53	0·62	1·55	1·80
0·55	0·76	1·55	1·91
0·62	1·0	1·64	1·75
0·66	0·88	1·70	1·9
0·8	1·22	1·87	1·98
0·8	1·07	1·95	2·05
0·85	0·88	2·62	3·70
0·96	1·38	3·67	5·1
0·97	1·5	4·83	8·5
1·0	1·2	5·0	12·0
1·05	1·7	5·2	9·5
1·05	1·35	5·2	6·84
1·1	1·68	5·25	8·5
1·1	1·18	5·3	7·5
1·13	1·55	5·4	7·8
1·13	1·65	6·0	8·91
1·15	1·75	6·0	7·5
1·15	1·47	6·0	7·72
1·20	1·35	6·25	9·2
1·20	1·82	7·0	9·0
1·25	1·45	10·0	21·5
1·26	1·65	10·0	15·0
1·30	1·38	11·0	18·8

all of which the 606 $m\mu$ band is well shown, discloses the same general trend over the table as a whole, but many minor discrepancies. In Table II are given the data for 19 oils in which the intensity of the 606 $m\mu$ band is unusually low in relation to the ultra-violet intensity of absorption.

If now a selection from both Tables is made of a number of oils practically indistinguishable as regards ultra-violet absorption, it will be seen how very far from real constancy the blue colour intensities can be.

The conclusion is inevitable that either the over-all experimental error of the physical assay is 250 %, or the 606 $m\mu$ chromogen and the substance

Table II. *Cod-liver oils in which the value of* $E^{1\%}_{1\,cm.}$ *328*mμ *is greater than the value of* $E^{1\%}_{1\,cm.}$ *606*mμ.

328 $m\mu$	606 $m\mu$	328 $m\mu$	606 $m\mu$
0·14	0·05	1·15	1·0
0·50	0·50	1·15	0·72
0·57	0·50	1·22	1·0
0·58	0·58	1·35	1·25
0·62	0·35	1·37	1·22
0·75	0·57	1·38	1·25
0·75	0·45	1·8	1·78
0·85	0·81	1·86	1·53
0·85	0·66	1·89	1·62
1·07	0·72		

Table III. *Cod-liver oils closely alike as regards ultra-violet absorption but differing in blue value.*

$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
1·07	0·72	1·15	0·72
1·1	1·18	1·15	1·00
1·05	1·75	1·15	1·47
1·05	1·70	1·15	1·75
1·1	1·68		

responsible for the 328 $m\mu$ maximum are not simply related. The discrepancy is vastly greater than any experimental error of which we are aware in the physical measurements.

It must however be emphasised that these data embody the results of a very large number of tests on materials ranging from the purest medicinal oil to very crude fish-liver oils of the cattle feeding variety.

A careful inspection of our data on concentrates reveals a similar state of affairs.

Table IV. *To illustrate the more rapid increase in the 620*mμ *band than in the 328*mμ *as richer and richer concentrates are compared.*

$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 620 $m\mu$	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 620 $m\mu$
13	22·4	267	650
22	33	275	400
85	174	357	772
112	274	370	950
113	200	500	1140
115	213		

Judging from the mass of data it is impossible to regard these two sets of figures on the blue colour and the ultra-violet absorption respectively as measuring the concentration of the same substance.

We may now consider the data for oils and concentrates in which the intensities of absorption in the colour test have been measured both at 572 and 606 $m\mu$ (583 and 620 $m\mu$ for concentrates).

From Table V it will be seen that there is a much closer parallelism between the intensities at 572 and 328 $m\mu$ than is to be found between those of the 606

Table V.

Material	Ultra-violet absorption		Colour test	
	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ min.	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
Codling-liver oil (*a*)	0·125	0·11	0·12	0·06
,, (*b*)	0·22	0·20	0·20	0·13
Cod-liver oil 1	0·38	0·38	0·33	0·33
,, 2	0·55	0·45	0·54	0·54
,, 3	0·55	0·52	0·54	0·62
,, 4	0·55	0·55	0·37	0·47
,, 5	0·57	0·50	0·50	0·50
,, 6	0·57	0·50	0·50	0·58
,, 7	0·67	0·57	0·50	0·61
,, 8	0·75	0·65	0·57	0·57
,, 9	0·82	—	0·83	0·25
,, 10	0·80	—	0·70	0·92
,, 11	0·85	0·55	0·68	0·85
,, 12	0·85	—	0·70	0·88
,, 13	0·95	—	0·92	1·14
,, 14	0·95	0·75	0·81	1·20
,, 15	1·03	0·80	1·02	1·35
,, 16	1·1	—	1·0	1·18
,, 17	1·15	0·95	1·25	1·35
,, 18	1·27	1·05	1·41	1·45
,, 19	1·30	—	1·28	1·50
,, 20	1·37	0·9	1·40	1·22
,, 21	1·40	—	1·37	1·25
,, 22	1·50	0·7	1·65	1·51
,, 23	1·60	—	1·20	1·65
,, 24	1·70	—	1·85	1·90
,, 25	1·65	0·55	1·45	1·92
,, 26	1·80	—	1·43	1·78
,, 27	1·86	1·60	1·74	1·53
,, 28	1·87	—	2·32	1·98
,, 29	1·89	0·50	1·86	1·62
,, 30	1·95	1·0	1·80	2·05
,, 31	4·3	—	3·81	4·76
Fish-liver oil 1	6·2	2·5	5·83	8·91
,, 2	3·67	2·0	3·4	5·1
Halibut-liver oil	11·5	4·0	23	28
Liver oil	4·83	—	4·4	8·5
Concentrate	57·5	—	59·4	101·2
,,	267·0	170	286	650

and 328 $m\mu$ bands. This fact is fully borne out in Table VI, which is merely a representative selection of the data which have been accumulated.

Table VI. *The trend of spectroscopic data with increasing concentration of vitamin A.*

	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 to 583 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 to 630 $m\mu$
Cod-liver oils	1·0	1·0	1·35
Rich fish-liver oils	6·25	5·8	9·0
Concentrates 1	55	60	100
,, 2	267	286	650
,, 3	370	400–500	950

The results of a comparison of biological assays made in London with similar spectroscopic data, support the view which is emerging from this work, namely that the 572 $m\mu$ (583 $m\mu$) chromogen is identical with the substance

responsible for the 328 $m\mu$ band and is in fact vitamin A, whereas the 606 $m\mu$ (620 $m\mu$) band has been recorded at from $\frac{1}{3}$ to 3 times the intensity of the 328 $m\mu$ band, a total variation of the order 10:1, whilst the gross variation for the comparison of the 572 $m\mu$ (583 $m\mu$) intensity at the 328 $m\mu$ band is less than 2:1.

The use of the 328 $m\mu$ band as a quantitative criterion of vitamin A involves the measurement of the gross absorption at 328 $m\mu$, a magnitude theoretically including:

(i) the true contribution of the 328 $m\mu$ band, *plus*

(ii) the contribution of the materials always present in cod-liver oils *etc.*, *i.e.* the normal fatty glycerides and the cholesterol, *plus*

(iii) the contribution of the variable and occasional non-vitamin A constituents, *e.g.* free acids and other impurities.

We have hitherto [Morton and Heilbron, 1928; Drummond and Morton, 1929] used a correction term of 0·13 to 0·22 ($\log I_0/I$ for a 1 % solution and a 0·1 cm. cell) for the joint effect of (ii) and (iii) in average medicinal cod-liver oils. Further experience on a wider range of materials has convinced us that the absorption of the fatty glycerides and the cholesterol is negligibly small, and that the excessively high values for the absorption at 328 $m\mu$ arise from the presence of impurities which vary from sample to sample. The correction of the 328 $m\mu$ intensity has therefore been abandoned as too uncertain in its operation. On the whole there is very striking agreement, considering the adverse conditions, between the intensity of absorption at 328 and 572 $m\mu$ (583 $m\mu$). This implies that the molecular extinction coefficient of the 328 $m\mu$ absorption band found in alcoholic solutions is approximately the same as that of the blue pigment band at 572 $m\mu$ resulting from the interaction of antimony trichloride with the chromogen. It is not uncommon in the study of the absorption spectra of related organic compounds to meet cases of bodily wave-length displacement of an absorption band without great change in extinction coefficients as we pass from one substance to others. That such a result is found with liver oils perhaps gives independent support to the view that the ultra-violet band and the 572 $m\mu$ band in the colour test are properties of vitamin A and a derivative respectively.

It may be stated that we have carefully explored the possibility that the intensity of absorption at 328 $m\mu$ is a summation of two factors, one the absorption arising from the 572 $m\mu$ chromogen and the other that due to the 606 $m\mu$ chromogen. This enquiry indicates that the substance responsible for the 606 $m\mu$ band with antimony trichloride is very transparent to light of the wave-lengths near 320–330 $m\mu$.

The views we have advanced necessitate some caution in using the Lovibond technique as a quantitative method of assay. The blue colour is complex, arising from at least two apparently independent absorption bands; in oils we find with antimony trichloride a band in the yellow-green (572 $m\mu$), and a band in the orange (606 $m\mu$), whilst with concentrates the bands are in the

yellow (583 $m\mu$) and the orange-red (620 $m\mu$). Complementary to these bands we have colours which may be roughly described as ranging from violet-blue to greenish-blue. Matching such colours against glasses coloured blue with cobalt gives only a very approximate evaluation of intensities because the procedure rests upon the subjective matching of physically distinct phenomena. Moreover the two absorption bands both count in the matching, so that the colorimetric procedure results in a rough evaluation of the oils as regards the combined quantities of two chromogens present. In actual practice the Lovibond technique gives better results than would be antipicated from this argument, owing to the fact that in a fair percentage of cod-liver oils the relative intensities of the 606 $m\mu$ and 572 $m\mu$ absorption bands are near 1·35:1. A prolonged search has however, as we have shown, made clear that this ratio is by no means to be relied upon in all cases.

We have further obtained definite evidence [Heilbron, Gillam and Morton, 1931] that the concentration of the 606 $m\mu$ chromogen can increase spontaneously without any accompanying change in the 572 $m\mu$ chromogen or the 328 $m\mu$ absorption intensity. Finally we have been able to achieve this increase in the laboratory, so that the existence of two independent chromogens in liver oils seems to be established.

Conclusions.

Liver oils contain two chromogens which, with antimony trichloride, give coloured substances with absorption maxima at 606 $m\mu$ and 572 $m\mu$ respectively. In concentrates these maxima are displaced to 620 $m\mu$ and 583 $m\mu$. A comparison of ultra-violet absorption spectra with spectroscopic data on the colour test discloses (i) that the parallelism between the intensity of the 606 $m\mu$ band and the intensity of the 328 $m\mu$ band breaks down so seriously in extreme cases as to render it improbable that the 606 $m\mu$ chromogen is vitamin A; (ii) that the 572 $m\mu$ chromogen and the substance responsible for the 328 $m\mu$ band are probably identical, (iii) that the blue colour for rich oils and concentrates is often much deeper than would be expected on the basis of correlation between blue colour and ultra-violet absorption. Hence it is concluded that the matching of blue colours with Lovibond glasses, though it may act as a rough guide to vitamin A potency is theoretically unsound.

REFERENCES.

Drummond, Ahmad and Morton (1930). *J. Soc. Chem. Ind.* **49**, 291 T.
—— and Morton (1929). *Biochem. J.* **23**, 785.
Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.
Moore (1930). *Biochem. J.* **24**, 3, 694.
Morton and Heilbron (1928). *Biochem. J.* **22**, 987.
—— —— and Thompson (1931). *Biochem. J.* **25**, 20.

CXLVIII. SPECIFICITY IN TESTS FOR VITAMIN A. A NEW CONCEPTION OF THE CHROMOGENIC CONSTITUENTS OF FRESH AND AGED LIVER OILS.

By ISIDOR MORRIS HEILBRON, ALBERT EDWARD GILLAM and RICHARD ALAN MORTON.

From the Department of Chemistry, The University of Liverpool.

(*Received July 3rd, 1931.*)

Evidence is accumulating to the effect that the vitamin A of liver oils is a colourless, or almost colourless substance, characterised by a broad continuous band of selective absorption in the ultra-violet, with a maximum near 328 $m\mu$. The vitamin acts as a chromogen in the sense that with antimony trichloride it gives rise to one and possibly more than one absorption band in the visible.

In the earlier stages of the reaction, the colour arises to a considerable extent from a pronounced absorption band in the yellow-green, the maximum being near 583 $m\mu$ but suffering a variable displacement towards 565–575 $m\mu$ in the presence of certain little-understood, unsaturated substances present in liver oils. The evidence indicates that associated with vitamin A is a substance, which, unlike the vitamin, is highly transparent to light of wave-lengths near 328 $m\mu$ but shares with it the property of acting as a chromogen towards antimony trichloride, giving rise in this case to a coloured compound absorbing maximally in the orange at 620 $m\mu$, the band again undergoing a displacement (to 600–610 $m\mu$) in the presence of certain unsaturated substances of unknown constitution normally present in fish-liver oils.

The two chromogens at present appear to be distinct and separate entities, but their occurrence together, and in quantities not widely different as regards order of magnitude makes it necessary to investigate the possibility of kinship in origin or function.

It is thus clear that the colour test with antimony trichloride occupies a position of cardinal importance in the present phase of development in our knowledge of vitamin A. It is therefore worth while to note the characteristics of the ideal colour test so that the antimony trichloride test may be seen in proper perspective.

The simplest case, in which subjective or objective colorimetry reaches its highest accuracy, is that of solutions containing a single absorbing solute which rigidly conforms with the Lambert-Beer absorption law[1]. The solutions

[1] In any two solutions exhibiting identical absorption of light $c_1 = c_2 d_2/d_1$, where c_1, c_2 are concentrations and d_1, d_2 are thicknesses.

to be compared should not differ in respect of the proportion of any colourless constituents able to depress or enhance the visible absorption of the coloured solute.

The two solutions under comparison should thus exhibit identical spectral absorption curves when the thicknesses are so adjusted that the number of molecules absorbing visible rays is the same in both light paths. Under these circumstances the matching of both solutions separately against coloured glasses is not open to serious objection.

Some of the characteristics of the antimony trichloride colour test, especially as applied to sources of vitamin A may now be noted.

(i) Visible colorations varying from red to blue are given with a wide variety of substances ranging from carotenoids to sterols, and including besides vitamin A, highly unsaturated polyene acids such as the crocetins. There is therefore, in a fundamental sense, nothing very specific about the colour test.

(ii) The reagent itself is a source of difficulty. Ordinary B.P. chloroform contains alcohol, which tends to inhibit the production of the maximum intensity of colour. Specially purified chloroform[1] dissolves appreciably less anhydrous antimony trichloride than B.P. chloroform, and relatively small changes in concentration of antimony trichloride appreciably affect the absolute intensity of the blue colour. The reagent as sometimes used is unstable towards light and moisture, and if kept for a few weeks, even in dark brown bottles, tends to deposit a heavy oily liquid.

(iii) As a criterion of vitamin A, the colour test is applied to a wide variety of substances, including mammalian-liver fats, butter extracts, crude and refined oils from fish-livers of many different species, and non-saponifiable extracts at various stages of concentration and diluted in various ways. The "environment" of vitamin A may therefore include sterols, carotenoids and many different saturated and unsaturated substances. It is rarely possible to feel confident that the colour test is free from interference arising either as a result of the presence of chromogens other than vitamin A or as a result of some degree of inhibition. Even restricting attention to one source such as "cod-liver oil," the variations in ordinary physical and chemical properties between individual samples are often very great, quite apart from variations in vitamin A potency. The conditions are so adverse in the sense of a lack of constancy in the environment of vitamin A that the only surprise which need be felt is that the colour test responds as well as it is in fact found to do.

(iv) The colour is transient; not only does it fade, but the rate of fading, the succession of colour changes and the more or less final colour are all subject to considerable variations. There is no doubt that, as at present available (*e.g.* in concentrates), vitamin A itself does not form a stable compound with antimony trichloride and that a succession of secondary actions occurs. Thus the two chromogens which at present appear to be of greatest interest

[1] *Cf.* Report of Cod-Liver Oil Colour Test Sub-Committee, March, 1931, Pharmacopoeia Commission, General Medical Council.

give coloured substances with initial maxima varying from 565 to 585 $m\mu$ and 600 to 624 $m\mu$ depending on the environment. Maxima at 535, 495 and 465 $m\mu$ have all been recorded as the solutions faded. Evidence of other chromogens is provided by the appearance in certain cases of bands at 635, 645, 656, 680 and 690 $m\mu$. All these maxima have been found in fish-liver oils and concentrates. The chromogenic properties of substances in the carotenoid and sterol groups are equally complicated.

(v) The colour test is very sensitive to mass action. In most cases the use of half-strength reagent effects an enormous reduction in the intensity of the blue colour. Even this is not a simple case of mass action, for as we shall show later, it is evident from the spectroscopic data that different chromogens are affected differently by a change in concentration of the reagent.

(vi) It has recently been shown (*cf.* preceding papers) not only that there are two chief chromogens in liver oils, but that the relative proportions may sometimes differ so widely as to effect a gross qualitative change in the shape of the spectral absorption curves characterising the solutions. Thus the 572 $m\mu$ maximum is sometimes vastly stronger than the 606 $m\mu$ maximum and *vice versa*.

In view of the complexity of the phenomena associated with the colour test, the experimental methods must be as searching as possible. Colorimetry, using standard glasses, is of distinct value as a simple and convenient way of expressing colour intensities with some approach to accuracy. For research purposes colorimetry is however of limited value unless frequent parallel spectroscopic determinations are carried out.

If the spectral absorption curves for the blue solutions were always the same, it is indisputable that objective colorimetry using photoelectric cells and recording instruments would provide a highly accurate method for measuring intensities, especially if used in conjunction with a monochromator or a filter capable of eliminating all but a narrow strip of the spectrum. Photoelectric spectrophotometry is however less useful than visual methods for examining the spectra of the blue solutions with which we are concerned, because of the need for rapid detection of variable maxima in the absorption curves. With the Hilger-Nutting spectrophotometer a blue solution can be seen at once to be either normal or abnormal, the wave-length of maximal absorption can be selected, and the intensity of absorption determined, all in a few seconds. The photoelectric technique is unsuitable for exploring spectra, its usefulness is for measuring the intensity of absorption at a given wave-length quantitatively. Unfortunately, each blue solution must be examined visually before the wave-length suitable for the photoelectric method can be selected.

Technique. The following is a description of the technique used in these laboratories.

The reagent (a saturated solution of antimony trichloride in purified chloroform) is kept in the apparatus shown in Fig. 1. The oil or concentrate

is dissolved in pure chloroform, the concentrations chosen being 20 % for an average cod-liver oil and 0·2 % for a concentrate about 100 times as rich as an oil. The solution is placed in the modified pipette shown in Fig. 2, so that

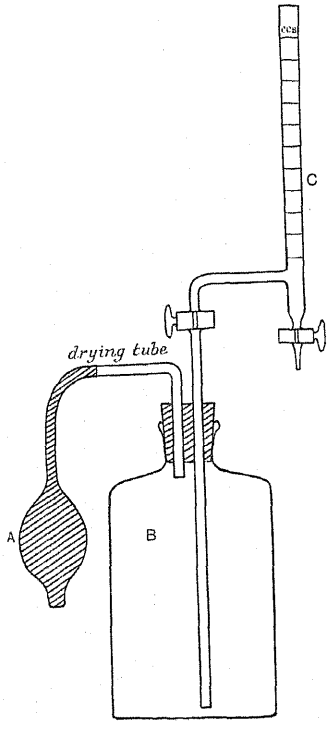


Fig. 1.

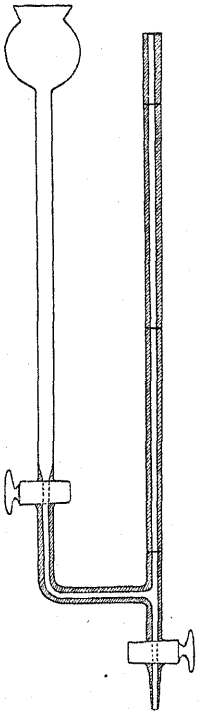


Fig. 2.

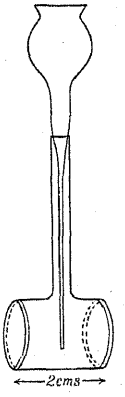


Fig. 3.

Fig. 1. Type of apparatus used for storage of antimony chloride in chloroform.
A. Rubber bulb with valve. B. Stock bottle of solution. C. 10 cc. burette.
A drying-tube can be placed between the bulb and the air inlet if the reagent is to be kept in this apparatus.

Fig. 2. Form of pipette used for measurement of chloroform solutions of cod-liver oil (2 cc. graduated in tenths).

Fig. 3. Type of fused quartz cell used for spectroscopic examination of antimony chloride-vitamin A blue colours.

frequent pipetting of chloroform is avoided. We are indebted to Mr H. Rogerson for these simple and efficient methods.

The volume of solution used is 0·3 to 0·6 cc., generally 0·5 cc. This is run into a test-tube, and 5 cc. of antimony trichloride are added. The mixture is at once poured into a 2 cm. fused quartz cell with plane parallel fused-on end plates (*cf.* Fig. 3) which is already mounted in position in the Hilger-Nutting spectrometer. The positions of absorption maxima are first ascertained. The cell is then emptied and the attachment at the spectrometer eye-piece adjusted so that a narrow strip of the spectrum on either side of the maximum is isolated. A fresh mixture is then prepared and the intensity of absorption at the maximum is determined. A number of readings are always made on

fresh mixtures, usually by two observers, and the average values reduced to a basis of 1 %, 1 cm. The ultra-violet absorption spectra are determined in the ordinary way, the results being expressed in terms of $\log I_0/I$ (E) at 328 $m\mu$ for a 1 % solution and a 1 cm. cell, no corrections being used unless the persistence of the band is abnormally small, in which case the fact of correction is noted. The numerical values of $E^{1\%}_{1\,\text{cm.}}$ 572 and $E^{1\%}_{1\,\text{cm.}}$ 328 $m\mu$ are in most cases identical within experimental error. We have therefore adopted $E^{1\%}_{1\,\text{cm.}}$ 1·0 as a spectroscopic unit. This value is on the average equal to a blue value (Carr-Price) of 12·5 Lovibond.

It has been shown [Morton, Heilbron and Thompson, 1931] that the blue solutions with liver oils or concentrates may exhibit very marked qualitative differences in absorption spectra, the maximum being liable to occur at 572 $m\mu$ or 606 $m\mu$. Gillam and Morton [1931] on the basis of the comparison of ultra-violet absorption and spectroscopic data on the colour test have shown that an almost exact linear relationship obtains between the intensity of the 328 $m\mu$ maximum and the 572 $m\mu$ chromogen, whereas the relationship between the 328 $m\mu$ band and the 606 $m\mu$ band exhibits a sufficient number of serious discrepancies from linearity to impugn the hypothesis of a common origin. It has also been shown [Coward, Dyer, Morton and Gaddum, 1931], on the basis of the latest and fullest comparison of biological assays with physico-chemical data, that the relative intensities of the 328 $m\mu$ and 572 $m\mu$ maxima provide trustworthy criteria of potency, whereas the values based on the 606 $m\mu$ maximum and on the Lovibond tintometer readings exhibit many anomalies. Further, Lovern and Morton [1931] in studying the oil from the livers of the monk or angler fish observed instances in which the concentration of the 606 $m\mu$ chromogen relatively to the 572 $m\mu$ chromogen was abnormally low, but in which the intensity of the 328 $m\mu$ ultra-violet band corresponded very closely with the intensity of absorption at 572 $m\mu$. The same authors also encountered cases of codling- and cod-liver oils in which similarly low concentrations of the 606 $m\mu$ chromogen were found, although the 572 $m\mu$ chromogen occurred strongly and in amount agreeing with the intensity of the ultra-violet band. We now desire to submit fresh evidence pointing still more definitely to the existence of two distinct chromogens in liver oils.

The claims made by Mittelmann [*cf.* Lovern, Creed and Morton, 1931] seemed to be of special interest as soon as misgivings arose as to the validity of a strict correlation between the unresolved blue colour test and the ultra-violet selective absorption. It became evident in testing the Mittelmann process that the particular oils studied showed an increased chromogen content on standing. The most natural hypothesis to advance as an explanation of such a spontaneous change was that of oxidation. It seemed therefore desirable to ascertain whether the process could be accelerated. Accordingly the following methods were tried.

(i) A current of ozonised oxygen was passed through the oil in the cold for a short time.

(ii) The oil was shaken with about one-fifth its volume of a concentrated solution of hydrogen peroxide for 10–15 minutes, the excess of peroxide washed away with water and the oil dried with anhydrous sodium sulphate and filtered.

(iii) The oil was dissolved in chloroform, a solution of benzoyl peroxide added and the mixture left to stand in the cold for 10–15 minutes. A small quantity of this solution was then used for the antimony trichloride colour test.

(iv) The oil was treated with barium peroxide.

(v) The oil was treated with sodium peroxide.

Method (iv) proved useless and method (v) ineffective in the absence of water, but very striking results were obtained with the other three reagents. The oils investigated were remarkable for the abnormally high $572\,m\mu/606\,m\mu$ intensity ratio, absorption at the latter point being quite feeble. As will be seen from Table I, the treatment with ozone results in a very large increase in the measurable concentration of the $606\,m\mu$ chromogen, the $572\,m\mu$ chromogen and the ultra-violet absorption being but little changed (*cf.* Fig. 4).

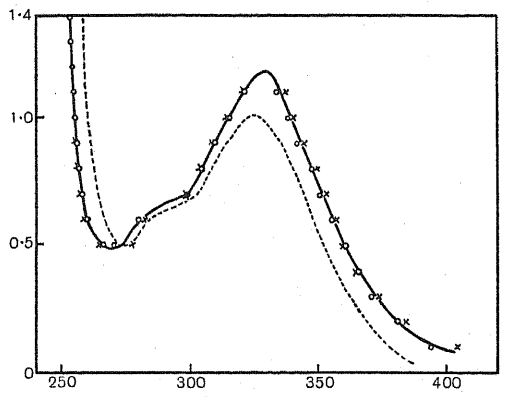


Fig. 4.

—o—o— Steamed C.L.O. 1·5 % (1 cm.).
×—×—× Steamed C.L.O. ozonised 1·5 % (1 cm.).
- - - - - - - Mittelmannised oil from 10 tins 1·5 % (1 cm.).

The enhanced concentration of the $606\,m\mu$ chromogen is well shown in the case of the monk-liver oil. This oil had been obtained a very short time previously by ether-extraction of the livers and had been stored in a glass bottle under ordinary laboratory conditions. The codling oils were obtained by the Mittelmann process, the fresh livers having been sealed in evacuated tins and autoclaved at 120° for 2 hours. The oils were examined within 2–3 days of the fish being caught. Here again the $572\,m\mu$ chromogen predominated to a marked extent over the $606\,m\mu$ chromogen, but the ultra-violet intensity was in good agreement with the higher value. Ozonisation or treatment with peroxides resulted in a very considerable increase in the $606\,m\mu$ chromogen. These experiments were repeated, fresh tins being opened for the purpose. The results proved to be reproducible (*cf.* Table I).

Table I. *The action of O_3, H_2O_2, etc., on very fresh liver oils.*

Oil	Remarks	Ultra-violet test $E^{1\%}_{1\,cm.}$ 328 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 572 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 606 $m\mu$
Monk-liver oil (pigmented)	As received	0·82	0·825	0·25
	Treated ozone	0·95	0·69	0·96
	Treated H_2O_2	—	0·82	0·96
	+ benzoyl peroxide	—	0·83	1·00
Codling-liver oil (small livers)	As received (liver in tins)	0·12	0·12	0·06
	Same. Next day	0·16	0·12	0·176
Codling-liver oil (larger livers)	As received	0·22	0·21	0·13
	+ O_3. 5 minutes	—	0·19	0·32
	+ O_3. 10 ,,	—	0·215	0·29
	+ O_3. 20 ,,	—	0·18	0·31
	+ O_2. 20 ,,	—	0·19	0·13
Codling-liver oil (larger livers)	As received	0·19	0·18	0·125
	+ O_3. 1 minute	—	0·17	0·14
	+ O_3. 3 minutes	—	0·175	0·20
	+ O_3. 5 ,,	—	0·175	0·25
	+ O_3. 10 ,,	0·19	0·17	0·27
	Treated H_2O_2	0·21	0·175	0·32
Cod-liver oil	As received	0·73	0·76	0·355
	+ O_3. 20 minutes	0·73	0·59	0·93
Cod-liver oil	As received	0·69	0·72	0·39
	+ O_3. 30 minutes	0·73	0·72	0·93
	After 11 days	0·75	0·75	1·00

Later tests showed that the unusual preponderance of the 572 $m\mu$ chromogen was not due to the Mittelmann process *per se* (*cf.* Lovern, Creed and Morton, 1931), since, using the same livers, the 572 $m\mu$ chromogen predominated in the fresh steamed oil and in the oil from newly opened tins. It is significant however that a threefold increase in the 606 $m\mu$ chromogen was obtained on treatment with ozone, hydrogen peroxide or benzoyl peroxide.

On examining our records for cod-liver oils which when first tested had shown the 572 $m\mu$ chromogen preponderating over the 606 $m\mu$ chromogen, a number of these were discovered, and all were re-examined for ultra-violet absorption and by the antimony trichloride colour test. It was found that in each of these, after the oil had stood for several months, a significant change had occurred, the concentration of the 606 $m\mu$ chromogen having increased considerably. The ultra-violet absorption exhibited a very much smaller increase in intensity, whilst the 572 $m\mu$ band was also but little changed. Each oil was then subjected to treatment with ozone or hydrogen peroxide, and in some cases it was found that the "ageing" process could be carried still further. In other cases it was found that the ageing process had reached a maximum, and that further treatment with ozone or hydrogen peroxide merely resulted in the gradual destruction of both chromogens as evidenced by lessened absorption both at 572 and 606 $m\mu$ in the colour test and at 328 $m\mu$ in the ultra-violet absorption (Table II).

We have thus demonstrated for eight cod-liver oils, two codling-liver oils and at least one monk-liver oil that two chromogens can be detected in the oils. Spontaneous or induced changes, which may be oxidations, result in a

Table II. *Spectroscopic data on the spontaneous and the accelerated ageing of cod-liver oils.*

Oil	Remarks	Ultra-violet test		Colour test	
		$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ min.*	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
1	As received	1·37	0·90	1·40	1·22
	2 months later	1·42	0·95	1·33	1·20
	Treated O_3	1·40	—	1·41	2·12
	Treated H_2O_2	—	—	1·52	2·42
2	As received	1·86	1·66	1·74	1·53
	5 months later	2·0	1·73	1·90	2·55
	Treated H_2O_2	—	—	1·90	2·83
3	As received	1·87	1·26	2·32	1·98
	7 months later	2·25	1·53	2·01	2·75
	Treated H_2O_2 (overnight)	—	—	1·75	2·50
4	As received	1·90	0·67	1·86	1·62
	5 months later	2·03	0·80	1·95	2·33
	Treated H_2O_2	—	—	2·09	2·70
5	As received	1·52	0·72	1·62	1·51
	5 months later	1·60	0·95	1·52	2·02
	Treated H_2O_2	—	—	1·41	2·05
6	As received	1·07	—	1·07	0·72
	7 months later	1·17	—	1·14	1·75
7	As received	1·23	0·90	1·35	1·52
	Treated H_2O_2	—	—	1·32	1·90

* This is the intensity of absorption at the minimum but the wave-length is variable.

very considerable increase in one chromogen before any apparent change occurs in the quantity of the other chromogen. On the other hand, the ultra-violet absorption is not appreciably affected by the spontaneous or induced enhancement of the 606 $m\mu$ chromogenic value. It is however undesirable to generalise from these results, for we have examined the question whether all absolutely fresh oils contain less of the 606 $m\mu$ chromogen than of the 572 $m\mu$ chromogen, but by some spontaneous or induced change can be enriched in the 606 $m\mu$ chromogen. Definite evidence has been obtained to the effect that in a number of quite fresh oils the 606 $m\mu$ band not only predominates slightly over the 572 $m\mu$, but is present at its full intensity, ozonisation effecting no increase.

In our experience, an oil in which the 572 $m\mu$ chromogen predominates over the 606 $m\mu$ chromogen is somewhat unusual. The two chromogens are so far not demonstrably related and there is thus no reason to assume a constant ratio either for the 572 and the 606 $m\mu$ intensities or the 572 and the potential 606 $m\mu$ intensity. The lack of constancy in the ratios (Table III) further emphasises the independence of the two chromogens, although the differences are not so great as to preclude kinship.

In evaluating the characteristics of concentrates we have repeatedly noticed that the "blue value" increases much more rapidly than the intensity of absorption in the ultra-violet. This is probably analogous to an observation made by Smith and Hazley [1930].

Table III.

$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ (maximum attainable)	Ratio 606 : 572 $m\mu$
1·4	2·4	1·71 : 1
1·75	2·85	1·63 : 1
2·3	2·5	1·09 : 1
1·86	2·7	1·45 : 1
1·62	2·05	1·27 : 1
1·07	1·75	1·64 : 1
1·35	1·90	1·41 : 1
0·82	1·0	1·22 : 1
0·21	0·32	1·52 : 1
0·18	0·32	1·77 : 1
0·76	0·93	1·22 : 1
0·72	1·0	1·37 : 1

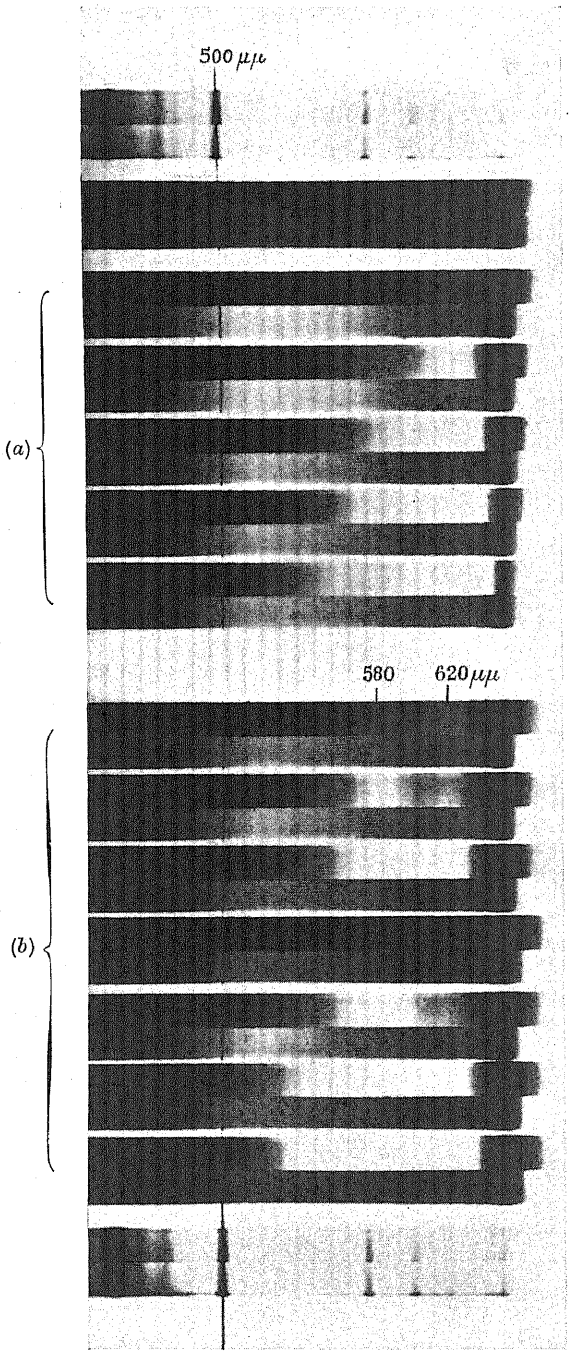
During the process of saponification the 572 $m\mu$ chromogen or its environment is changed so as to displace the maximum to 583 $m\mu$, but for this chromogen the increase in the intensity of absorption given by the colour test is exactly proportional to the degree of concentration effected. On the other hand, if the oil is such that the 606 $m\mu$ band fails to reach the maximum intensity possible for the oil, then for this second chromogen the increase in absorption surpasses that to be expected from the degree of concentration. In addition, a displacement to 620 $m\mu$ occurs. When concentrates are dissolved in either seal oil or whale oil the two revert to 606 and 572 $m\mu$, but in arachis oil no such reversion takes place. Clearly therefore the displacement of the maxima is somehow connected with the nature of the medium.

The results with concentrates are of importance in connection with the views of Norris and Church [1930, 1, 2]; *cf.* Smith and Hazley [1930]. In carrying out quantitative studies concerning the validity of the colorimetric test these authors were led to conclude that liver oils contain a saponifiable substance which tends to inhibit the production of the blue colour. Norris and Church [1930, 2] found that addition of oleic acid to concentrates produced the same inhibitory effect.

Separation of the two absorption maxima (colour test).

Spectroscopic examination of the blue solution obtained by the interaction of the antimony trichloride reagent and the majority of liver oils and concentrates shows the absorption curve to be unsymmetrical, with the sharper decrease of absorption occurring on the long-wave side of the maximum, Fig. 5 (*b*). With the anomalous oils, however, the curve is still unsymmetrical but in the opposite sense, Fig. 5 (*a*), the steep descent being now on the side of shorter wave-lengths. Now in most cases, starting from an oil giving higher absorption at 572 than at 606 $m\mu$, ozonisation or treatment with hydrogen peroxide effects so great an increase in the absorption at 606 $m\mu$ that the curve becomes of the type shown in Fig. 5 (*b*).

As we have seen, the interpretation advanced in this paper involves the view that an inflexion indicates the presence of a partially masked absorption



Aluminium spark.

Tungsten spark under water (control).

Cell	log I_0/I	Halibut oil cc.	$SbCl_3$ reagent cc.
2 cm.	0·8	0·05	5
,,	,,	0·10	5
,,	,,	0·15	5
,,	,,	0·20	5
,,	,,	0·25	5

Cell = 2 cm. log I_0/I = 0·8.

Halibut oil cc.	$SbCl_3$ reagent cc.	Interval min.	$SbCl_3$ reagent cc.
0·10	0·3	1	4·7
0·15	0·3	1	4·7
0·20	0·3	1	4·7
0·10	0·3	2	4·7
0·20	0·3	2	4·7
0·30	0·3	2	4·7
0·40	0·3	2	4·7

Aluminium spark.

(*a*) illustrates the appearance of one band in the colour test;
(*b*) ,, ,, ,, two bands ,, ,,

band. It would however be an advantage if the real existence of the 572 $m\mu$ band in normal oils could be demonstrated even more clearly. We have found this to be possible by following the procedure described below.

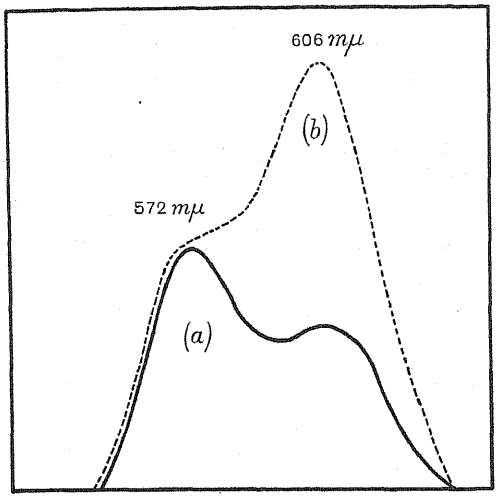


Fig. 5. Diagrammatic representation of the absorption spectra of the blue solutions produced with (*a*) certain fresh oils, (*b*) the same after spontaneous or induced ageing.

The usual quantity (0·5 cc.) of a solution of liver oil in chloroform is run into a test-tube and a small quantity (0·3 cc.) of the antimony reagent added. The solution is then allowed to stand for 1 minute and the remainder (4·7 cc.) of the reagent is then added and the solution examined spectroscopically. In nearly every case two separate absorption bands with maxima at 565–575 and 600–620 $m\mu$ can be seen very clearly. The intensity of the former band is practically the same as when the whole of the reagent is added at once, but the latter band is very much reduced in intensity.

It has been possible to photograph solutions treated in this way and the results are reproduced in Plate VI, whilst Fig. 6 shows a schematic representation of the absorption curves of the various coloured substances.

Table IV illustrates the results obtained by the alternative procedures.

Table IV.

			$E^{1\%}_{1\,cm.}$ 600–612 $m\mu$	$E^{1\%}_{1\,cm.}$ 572–575 $m\mu$
Halibut oil 1.	Procedure	(*a*)*	61·2	35·7
	,,	(*b*)	27·3	36·4
Halibut oil 2.	,,	(*a*)	101	58·5
	,,	(*b*)	62	50
Cod-liver oil 1.	,,	(*a*)	1·05	0·9
	,,	(*b*)	0·80	0·85
Cod-liver oil 2.	,,	(*a*)	2·75	2·2
	,,	(*b*)	1·87	2·1
Cod-liver oil 3.	,,	(*a*)	0·85	0·72
	,,	(*b*)	0·52	0·70

* (*a*) Normal procedure. (*b*) Preliminary addition of a small quantity of $SbCl_3$ reagent.

The striking phenomenon about the procedure involving the preliminary addition of a small quantity of reagent is that the reduction in intensity of

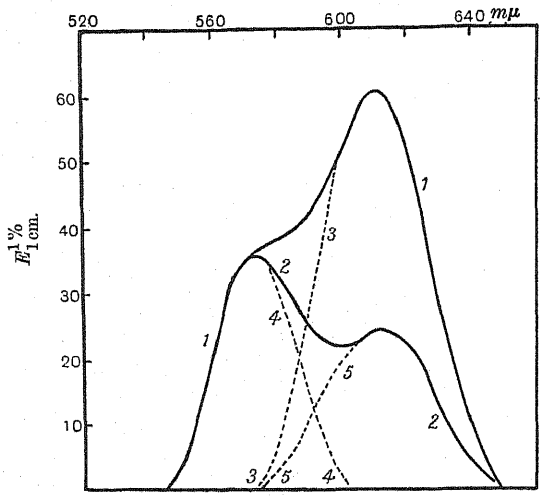


Fig. 6.

1. Schematic representation of absorption curves of the antimony trichloride blue colour with a halibut oil, measured on the freshly mixed solution, the reaction being carried out in the normal way (*a*).
2. Ditto, the reaction being carried out adding a small quantity of $SbCl_3$ reagent 1 minute before adding the bulk of the reagent (*b*).
3. Ditto, the 606 $m\mu$ band in case (*a*).
4. Ditto, the 572 $m\mu$ band in cases (*a*) and (*b*).
5. Ditto, the 606 $m\mu$ band in case (*b*).

1. Summation of 3 and 4. 2. Summation of 4 and 5.

the blue colour is substantially confined to the 606 $m\mu$ band, while the 572 $m\mu$ band is obtained at almost its full intensity.

Another but less satisfactory method whereby the two absorption bands can be manifested is to vary the concentration of antimony trichloride in the reagent.

Table V indicates the values obtained with a rich cod-liver oil.

Table V.

	$E^{1\%}_{1\,cm.}$ 606 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 $m\mu$
Saturated solution of antimony trichloride (in B.P. chloroform previously treated with anhydrous calcium chloride)	5·23	4·05
Two-thirds saturated reagent	3·9	3·6
Half saturated reagent	3·2	3·1

With the full strength reagent the 606 $m\mu$ maximum is shown very clearly and an inflexion is seen at 572 $m\mu$, but with the diluted reagents two bands are visible separately, although neither is present at its full intensity.

The reality of two absorption bands in the coloured solutions is thus established beyond question and the variations in the relative intensities of the

two bands under different conditions go a long way towards accounting for the non-linear colour-concentration curves which have been recorded by many investigators.

Discussion.

Without prejudicing the issue concerning vitamin A, the physical data lead us to postulate the following entities.

(i) A substance X, which gives rise to the ultra-violet absorption with its maximum at $328 m\mu$.

(ii) The chromogenic substance X' in oils which gives rise to the $572 m\mu$ maximum.

(iii) The chromogenic substance X'' present in concentrates giving rise to the $583 m\mu$ maximum.

(iv) The chromogenic substance Y' in oils responsible for the $606 m\mu$ maximum.

(v) The chromogenic substance Y'' of concentrates causing the band at $620 m\mu$ in the colour test.

(vi) A substance giving rise to an absorption band with a maximum near $280 m\mu$.

(vii) The substance or substances which react with antimony trichloride to give coloured compounds with maxima at 640–650 $m\mu$ and 690–700 $m\mu$.

Confining our attention for the present to the first five, the comparison of intensities of absorption at 328 and $572 m\mu$ for oils leads to the view that X and X' are identical. Similar comparisons using concentrates indicate that X and X'' are identical. This leads to the possibility that X' is an ester and X'' the alcohol derived from it on saponification. This suggestion does not however provide an explanation of the variability of the maximum in the colour test, because (*a*) in liver oils (*e.g.* halibut oils) which have much higher blue values than cod-liver oils there is a progressive displacement from 570 to $582 m\mu$, the very rich oils being chromogenically very like concentrates, and (*b*) when a concentrate is dissolved in seal oil or whale oil the maximum for the solution appears at 570, but if arachis oil is used it remains at $583 m\mu$.

Exactly similar considerations lead to the view that the $606 m\mu$ chromogen Y' is identical with the $620 m\mu$ chromogen Y'', the differences being due entirely to the displacing action of unsaturated substances. This does not however account for the apparent increase in the 606–620 $m\mu$ chromogen in certain oils on ozonisation or treatment with hydrogen peroxide. The following three possible explanations of the phenomena suggest themselves, but the task of ascertaining which is the true one promises to be unusually difficult.

(1) Vitamin A, which we may regard as the material responsible for the $328 m\mu$ maximum, is the chromogen responsible for both the 572–$583 m\mu$ band and the 606–$620 m\mu$ maximum. Antimony trichloride is known to form the double compounds:

$C_6H_4(CH_3)_2$, $SbCl_3$ $C_6H_4(CH_3)_2$, $2SbCl_3$ *etc.*,

so that there is nothing inherently impossible in the compounds $SbCl_3$, vitamin A; $2SbCl_3$, vitamin A.

(2) There are two independent chromogens *X* and *Y* present in the oil. The absolute amount of *Y* present is not increased by ozonisation *etc.*, but the reaction

$$Y + SbCl_3 \rightarrow SbCl_3, Y \text{ (blue substance)}$$

only occurs to its full extent in the absence of certain "inhibitors" which are eliminated by treatment with ozone or hydrogen peroxide.

(3) The quantity of *Y* present in an oil undergoes a real increase on ozonisation by virtue of the presence of a precursor substance, which is converted into *Y* either by spontaneous ageing or accelerated oxidation. Such a precursor, if it exists, must be chromogenically inert, or relatively so, and will absorb rays of wave-lengths near $328 m\mu$ comparatively feebly.

The explanation given under (1) involves that the "chromogens" are not capable of separation, it accounts at once for the somewhat startling fact that an animal depleted of vitamin A but supplied with carotene accumulates both "chromogens" in the liver, but it demands for the ozonisation experiments the removal of "inhibitor" substances, which before ozonisation can form complexes with antimony trichloride to such an extent as to modify profoundly the equilibria:

$$\text{Vitamin A} + SbCl_3 \rightleftharpoons SbCl_3, \text{ vitamin A.}$$
$$SbCl_3, \text{ vitamin A} + SbCl_3 \rightleftharpoons 2SbCl_3 \text{ vitamin A.}$$

The inhibitor substances must however remove antimony trichloride from the sphere of action of vitamin A without themselves forming coloured substances. The fact that changes in the concentration of the reagent markedly affect the relative intensities of the 572 and $606 m\mu$ bands is consistent with an explanation attributing a dominant rôle to mass action. The idea that both bands arise from vitamin A is attractive because of its avoidance of the complication of a new chromogen, but its weaknesses are patent in view of the very hypothetical nature of the essential "inhibitors" and the difficulty of reconciling it with the results of a preliminary addition of the antimony trichloride reagent. Finally, it would seem reasonable to expect the full development of the $606 m\mu$ band to be at the expense of the $572 m\mu$ band, and this is not realised.

The explanation given under (2) enables us to retain temporarily the validity of the $606 m\mu$ band as a possible criterion of vitamin A (although probably forcing us to abandon the $328 m\mu$ band) without postulating a threefold increase in potency on ozonisation. It again however compels us to attribute a very important rôle to the inhibitors about which so little is known. It minimises the possibility of a simple correlation between ultra-violet absorption or feeding tests and the colour value and gives no rational account of the apparent correlation between the 328 and $572 m\mu$ bands.

Explanation (3) seems on the whole to fit the facts best, but its consequences are serious. It involves the view that tintometric methods of assay can only be regarded as good approximations. It requires two new substances, a chromogen and a chromogen precursor, the absorption spectra of which in the ultra-violet have so far eluded our attempts to discover them. It fits in extremely well with the best biological assays to which we have access, but it raises the question of the function of the new chromogen and its precursor.

From whatever angle the new data are regarded a plurality of working hypotheses seems inevitable. Whilst we have frankly envisaged the difficulties, it is necessary to define our attitude. The most plausible interpretation leads us to suggest the following physico-chemical criteria for vitamin A.

(i) A colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near 328 $m\mu$, and (ii) giving with antimony trichloride a coloured substance characterised by a maximum in the yellow between 580 and 590 $m\mu$, whilst the molecular extinction coefficients at 328 and 580–590 $m\mu$ expressed in terms of vitamin concentration should be approximately equal.

These criteria are not free from a measure of uncertainty from the strictly numerical standpoint, because the change in environment involved in the preparation of rich concentrates displaces the chromogen from 572 to 583 $m\mu$, the vitamin itself remaining a minor constituent of even the richest concentrates so far obtained. Further concentration might result in a continuation of this shift in wave-length.

Again, antimony trichloride produces coloured substances with a wide range of sterol and carotenoid derivatives, and in many cases the absorption maxima are found in the yellow-red region of the spectrum. It would be by no means surprising if the 583 $m\mu$ maximum were found to be by itself non-specific for vitamin A. Again, an absorption maximum near 320–330 $m\mu$ in the ultra-violet has never been claimed as a specific test for vitamin A, it merely indicates a property of vitamin A. There would be nothing very unexpected in the discovery of a sterol or carotenoid derivative exhibiting both a maximum near 580-590 $m\mu$ with antimony trichloride and an absorption band very like that observed in liver oils, the substance being still distinct from vitamin A. It would however be much less probable to find the same relative intensities of absorption at 328 and 583 $m\mu$. The nearest approach to satisfying these criteria has been obtained with "dihydrocarotene" prepared by the method of Smith [1931]. This product, which is undoubtedly a mixture, gives selective absorption in the near ultra-violet and with antimony trichloride a blue solution exhibiting maxima at 583 and 648 $m\mu$ [Heilbron and Morton, 1931]. The interpretation of these results must await the examination of the separate constituents present in the mixture, both by biological and physico-chemical methods [*cf.* Drummond and Ahmad, 1931; Karrer, Euler and Hellström, 1931].

SUMMARY.

1. The ideal conditions for accurate colorimetric determinations are considered, and the actual characteristics of antimony trichloride as a colour-producing reagent are reviewed in detail.

2. In a considerable number of oils characterised in the colour test by predominance of the 572 $m\mu$ band over the 606 $m\mu$ band, a large increase in the intensity of the latter band can be obtained by treating the oil beforehand with ozonised oxygen, hydrogen peroxide or benzoyl peroxide.

Oils which initially showed an excess of the 572 $m\mu$ chromogen over the 606 $m\mu$ chromogen undergo a slow spontaneous ageing which results in a marked increase in the intensity of the 606 $m\mu$ band. Increases in the 606 $m\mu$ absorption are not at the expense of the 572 $m\mu$ chromogen and are not accompanied by similar increases in the latter, and the absorption at 328 $m\mu$ remains practically constant throughout.

3. The reality of the two chromogens and the possibility of recording the 572–583 $m\mu$ band even in oils and concentrates in which the 606–620 $m\mu$ chromogen predominates can be demonstrated visually and photographically by a modification of the ordinary procedure in the colour test, which suppresses or eliminates some of the 606 $m\mu$ chromogen.

4. The bearing of the data on the vitamin A problem is discussed in detail, and the most plausible criteria for the vitamin are stated.

5. The two main chromogens behave as if they were separate and distinct entities but a close genetic relationship seems highly probable.

We are indebted to the Department of Scientific and Industrial Research for a grant which has enabled Mr R. H. Creed to be of much service in the laboratory work.

REFERENCES.

Coward, Dyer, Morton and Gaddum (1931). *Biochem. J.* **25**, 1102.
Drummond and Ahmad (1931). *J. Soc. Chem. Ind.* **50**, 183 T.
Gillam and Morton (1931). *Biochem. J.* **25**, 1346.
Heilbron and Morton (1931). *J. Soc. Chem. Ind.* **50**, 183 T.
Karrer, Euler and Hellström (1931). *Svensk Kemisk Tidskrift*, No. 5.
Lovern and Morton (1931). *Biochem. J.* **25**, 1336.
—— Creed and Morton (1931). *Biochem. J.* **25**, 1341.
Morton, Heilbron and Thompson (1931). *Biochem. J.* **25**, 20.
Norris and Church (1930, 1). *J. Biol. Chem.* **85**, 477.
—— —— (1930, 2). *J. Biol. Chem*, **87**, 139.
Smith (1931). *J. Biol. Chem.* **90**, 597.
—— and Hazley (1930). *Biochem. J.* **24**, 1942.

CXLIX. THE PHYSIOLOGICAL FUNCTION OF VITAMIN B_1.

By BIRES CHANDRA GUHA[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 2nd, 1931.*)

More striking among the theories which have been proposed to explain the mechanism of action of "vitamin B" (using the old terminology) are those that connect the vitamin with biological oxidation processes. Some investigators have considered the vitamin to be concerned in cellular respiration in general, while others have claimed the existence of a definite relationship between the vitamin and certain aspects of metabolism. Some workers, however, have opposed the evidence on which such claims are based. *Post mortem* examination of avitaminous animals, while affording much valuable information, has not led to any definite knowledge as to the rôle that the vitamin plays in body economy. In some recent experiments with rats we observed that if the vitamin B_1-deficient animals were killed before they passed into the moribund condition, no specific lesions could be found in the internal organs, although the deprivation of vitamin B_1 was quite severe. These observations suggest that the gross abnormalities observed in the extreme stage of vitamin B_1 deficiency are most probably the secondary results of a primary disorder [*cf.* Drummond and Marrian, 1926].

The present investigation represents an attempt to throw some light on this problem.

Method.

All work was carried out with rats, most of which were of the black and white strain. They were kept in separate cages and 2 drops of a potent cod-liver oil were fed daily to each of them apart from the basal diet. All other supplements were also separately administered unless otherwise mentioned.

A. Vitamin B_1 and the protein/carbohydrate ratio of the diet.

The amylum-toxin theory originally proposed by Eijkman [1897, 1, 2], which was opposed by Grijns [1901, 1907] and later discarded, has more than once been revived in modified forms [Funk, 1914; Randoin and Simonnet, 1924]. Randoin and Simonnet, working with pigeons, considered that there was a definite relationship between the "antineuritic" vitamin and the carbohydrate content of the diet, while Hartwell [1925] and Reader and Drummond [1926] found that rats on high-protein diets required larger allowances of

[1] Travelling Fellow of the University of Calcutta.

"vitamin B." It was, however, subsequently shown by Hassan and Drummond [1927] that the corrective agent for high-protein diets present in marmite was a heat-stable substance and, therefore, presumably not vitamin B_1.

In the present experiments the effect of the same optimum and sub-optimum doses of a relatively concentrated preparation of vitamin B_1 on the growth of groups of young rats subsisting on diets of varying carbohydrate/protein ratios was studied. It was considered that if vitamin B_1 plays a specific part in carbohydrate metabolism, sub-optimum doses of vitamin B_1 might have a greater influence on the growth of young rats receiving less carbohydrate than of those receiving more. The deleterious effect of excess protein in the diet, if any, was sought to be corrected by Hassan and Drummond's technique of increasing the quantity of alkaline-autoclaved marmite in the daily ration. In order to keep the conditions comparable, however, the same quantity of alkaline-autoclaved marmite was incorporated in high-protein as well as in low-protein diets. The animals were allowed to eat *ad libitum*.

Two litters containing six piebald rats each were broken up as evenly as possible as regards weight and sex into groups of two.

The following diets were used:

Diet 1	%	Diet 2	%	Diet 3	%
Sucrose	25	Sucrose	50	Sucrose	75
Caseinogen	71	Caseinogen	46	Caseinogen	21
Salt mixture (McCollum)	4	Salt mixture (McCollum)	4	Salt mixture (McCollum)	4

The caseinogen used was the "light, white casein" of the British Drug Houses. 20 cc. of a 50 % solution of alkaline-autoclaved marmite (prepared by autoclaving a concentrated aqueous solution of marmite with baryta at p_H 9 for 1½ hours at 18 lbs. pressure and subsequently removing the baryta by sulphuric acid) were incorporated per 100 g. of the basal diet. The animals received cod-liver oil and the vitamin B_1 preparation separately.

Table I shows the diet and the dose of the vitamin B_1 preparation received by each group of animals.

Table I.

Group	Basal diet	Dose of vitamin B_1 preparation* (cc.)
A	1	0·075
B	2	0·075
C	3	0·075
D	1	0·15
E	2	0·15
F	3	0·15

* Obtained by extracting brewer's top yeast with 50 % aqueous alcohol, concentrating the extract *in vacuo*, precipitating with neutral lead acetate and removing the lead from the filtrate by hydrogen sulphide. The optimum dose of the concentrated solution was 0·15 cc., containing about 30 mg. organic material [see Guha, 1931, 1].

The growth curves, shown in Figs. 1–3, indicate that the need for vitamin B_1 is not diminished with a reduction in the carbohydrate content of the diet.

It will be observed that animals on the high-protein diet exhibit somewhat less vigorous growth than the others, though no definite signs of ill-health

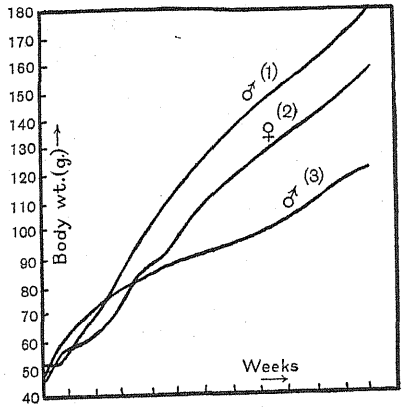


Fig. 1.

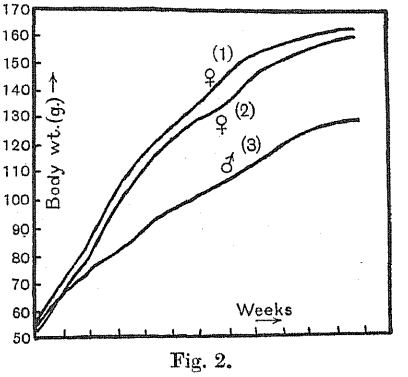


Fig. 2.

Fig. 1. Diet 1. Curves (1) and (2); dose = 0·15 cc. lead filtrate.
Curve (3); dose = 0·075 cc. lead filtrate.

Fig. 2. Diet 2. Curves (1) and (2); dose = 0·15 cc. lead filtrate.
Curve (3); dose = 0·075 cc. lead filtrate.

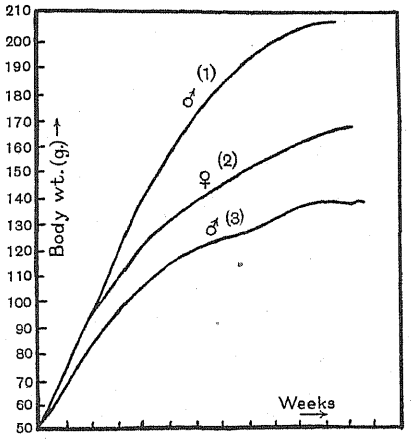


Fig. 3. Diet 3. Curves (1) and (2); dose = 0·15 cc. lead filtrate.
Curve (3); dose = 0·075 cc. lead filtrate.

were noticed. The high marmite content of these diets tended to give the rats slight diarrhoea during the first few days of experiment, which, however, rapidly cleared up.

B. The relation between vitamin B_1 and the nature of the dietary carbohydrate.

Considering the question of a possible relationship between vitamin B_1 and carbohydrate metabolism, it was of interest to investigate the effect of

a sub-optimum dose of vitamin B_1 on the growth of young rats receiving diets containing different carbohydrates. It has been observed [Campbell and Soskin, 1928; Campbell and Maltby, 1928; Rose, Giragossintz and Kirstein, 1929–30] that the administration of dihydroxyacetone and fructose by way of the small intestine is followed by a rise in blood-lactic acid, whereas aldoses like dextrose and galactose have no such effect. As Kinnersley and Peters [1929] have stated that avitaminosis B_1 in the pigeon results in the production of excess lactic acid, it was clearly desirable to know whether the requirement of vitamin B_1 would vary according as dextrose or fructose formed the carbohydrate in the diet, although the observations of Campbell and of Rose constitute no evidence that lactic acid is produced in the normal metabolism of ingested fructose.

In these experiments the following basal diets were used.

Table II.

Diet No.	Constituents of diet		
4	75 % dextrose;	21 % caseinogen*;	4 % salt mixture†
5	,, fructose;	,,	,,
6	,, sucrose;	,,	,,
7	,, galactose;	,,	,,
8	,, lactose;	,,	,,
9	,, maltose;	,,	,,

* B.D.H. "light, white casein." † McCollum's.

A litter of 8 young rats was split up into 3 groups, (*a*), (*b*) and (*c*), and fed as shown in Table II. Their growth curves are illustrated in Figs. 4–6, and their average weekly gains in weight are given in Table III.

Table III.

Rat No.	Sex	Group	No. of basal diet	Daily dose of vitamin B_1 (lead filtrate) cc.	Av. weekly gain in weight over 18 weeks g.
1	♂	(*a*)	4	0·075	3·8
2	♀	(*a*)	4	0·075	3·8
3	♀	(*a*)	4	0·075	3·7
4	♂	(*b*)	5	0·075	3·3
5	♀	(*b*)	5	0·075	4·2
6	♀	(*b*)	5	0·075	3·4
7	♀	(*c*)	6	0·075	3·8
8	♀	(*c*)	6	0·075	3·8

The vitamin B_1 administered (0·075 cc.) represents a sub-optimum dose. Each animal received the vitamin B_1 supplement, 1·5 cc. of 50 % alkalised marmite and 2 drops of cod-liver oil daily in a dish separately from the basal diet.

The figures of the growth rates given in Table III show that there is no special relation between the requirement of vitamin B_1 and the presence of dextrose, fructose or sucrose in the diet. When some of these animals were later (after about 25 weeks) deprived of vitamin B_1, those that were receiving fructose were found to decline at a somewhat slower rate than those receiving

dextrose or sucrose. This, however, could not be repeated when Kahlbaum's "crystallised laevulose" was used instead of the B.D.H. product. It is possible

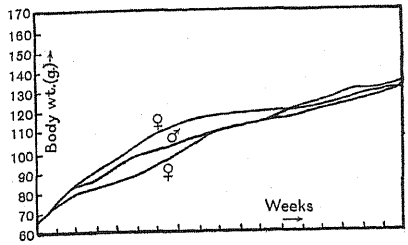


Fig. 4. Diet 4; dose = 0·075 cc. of lead filtrate.

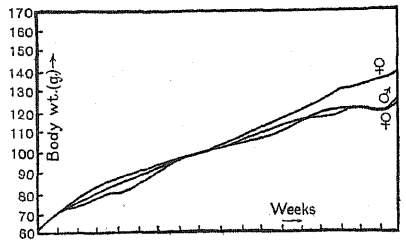


Fig. 5. Diet 5; dose = 0·075 cc. of lead filtrate.

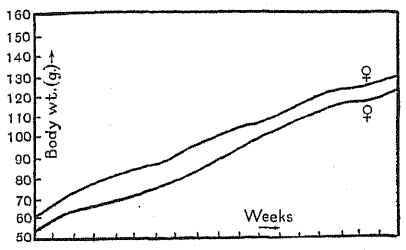


Fig. 6. Diet 6; dose = 0·075 cc. of lead filtrate.

that this latter carried traces of vitamin B_1, although it gave no perceptible precipitate with phosphotungstic acid.

When diets 7 and 8, containing galactose and lactose respectively as the sole sources of carbohydrate, were used, it was found that they caused a rapid decline of both young and adult rats. In the case of diet 7 final collapse could not be prevented even by incorporating whole dried yeast (5 %) in the diet. This phenomenon has been further investigated separately [Guha, 1931, 2].

Diet 9 containing maltose produced as good growth as the diet containing dextrose.

C. The relation between vitamin B_1 and dietary fat.

In the course of investigations on the effect of purified rations on the growth of rats, Evans and Lepkovsky [1929] made the striking observation that the presence of fat in the diet diminished the need of the rat for vitamin B_1. As the importance of this observation in relation to the suggested rôle of vitamin B_1 in carbohydrate metabolism is obvious, this question was investigated with reference to the following points.

(1) The effect of a sub-optimum dose of vitamin B_1 on the growth of young animals receiving diets containing (*a*) varying amounts of the same fat; (*b*) different fats in the same proportion; (*c*) varying proportions of fat but the same restricted amount of food every day.

(2) The effect of a complete deficiency of vitamin B_1 on rats subsisting on diets containing varying amounts of fat.

The following basal diets were used. The protein in each of the diets consisted of "light, white casein" (B.D.H.).

Table IV.

	Diet 12 %	Diet 13 %	Diet 14 %
Sucrose	65	35	10
Palm kernel oil	10	40	65
Caseinogen	20	20	20
Salt mixture (McCollum)	5	5	5

	Diet 24 %	Diet 25 %	Diet 26 %
Sucrose	65	35	10
Olive oil	10	40	65
Caseinogen	20	20	20
Salt mixture	5	5	5

	Diet 28 %	Diet 29 %	Diet 30 %
Sucrose	65	35	10
Lard	10	40	65
Caseinogen	20	20	20
Salt mixture	5	5	5

	Diet 44 Parts		Diet 45 Parts		Diet 49 Parts
Lard	50	Sucrose	50	Sucrose	70
Caseinogen	36	Caseinogen	36	Caseinogen	20
Salt mixture	4	Salt mixture	4	Salt mixture	4

Of these, diets 44, 45 and 49 were incorporated with 20 cc. of 50 % alkalised marmite per 100 g. of the diet. The caseinogen content in diets 44 and 45 was raised in order to bring the nutritive ratio approximately to 1:3 and thus to make them more balanced than the other high fat diets [see Evans and Lepkovsky, 1929]. Diets 44 and 49 were made up so as to resemble Evans and Lepkovsky's diets 559 and 542 respectively. When the other basal diets were used, 1 cc. of alkalised marmite was fed separately every day. This dose was considered sufficient as 2 cc. gave no better growth. Two drops of cod-liver oil were, as usual, fed separately to each rat. Vitamin B_1, whenever fed, was administered separately in a sub-optimum dose (0·05 cc. of a lead filtrate preparation from brewer's yeast, different from that used for work described in the preceding sections).

Litters were evenly broken up as regards weight and sex and litter-mates used for the same batch of experiments. Some animals were kept under observation for periods exceeding 25 weeks. Figs. 7–9 illustrate some of the results obtained. Food intake records, which were kept for a period of 3 months, show that in general the gain in weight goes hand in hand with the food consumption. It will be observed that while olive oil and palm kernel oil do not give any definite indication of a sparing action on vitamin B_1 when the animals are receiving a sub-optimum dose of vitamin B_1, lard appears to have a definite effect. The decline in weight of rats completely deficient in vitamin B_1 is also slower when lard is present in the diet.

Four rats which were receiving diets 28 and 29, containing respectively 10 and 40 % lard, supplemented by a sub-optimum dose of vitamin B_1, were

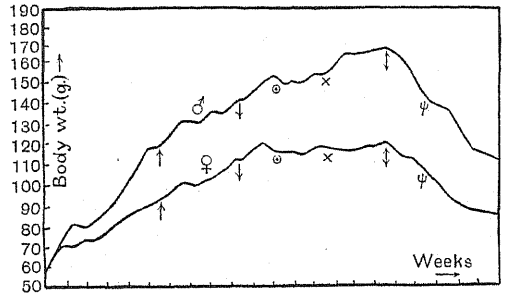


Fig. 7. Dose of vitamin B_1=0·075 cc. of lead filtrate throughout except as stated below; basal diet 12 from beginning of experiment; ↑ diet 24; ↓ diet 28; ⊙ restricted food intake; × food *ad libitum*; ↕ diet 45, B_1 supplement stopped; ψ diet 49 (no B_1).

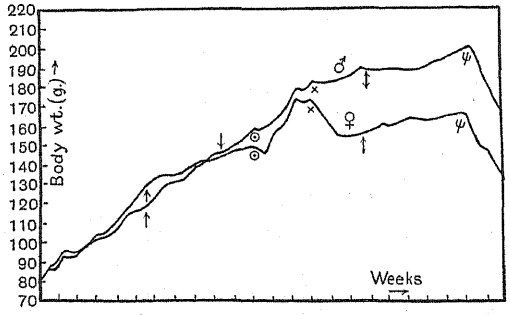


Fig. 8. Dose of vitamin B_1=0·075 cc. of lead filtrate throughout except as stated below; diet 13 from beginning of experiment; ↑ diet 25; diet 29; ⊙ restricted food intake; × food *ad libitum*; ↕ diet 44, B_1 supplement stopped; ψ diet 49 (no B_1).

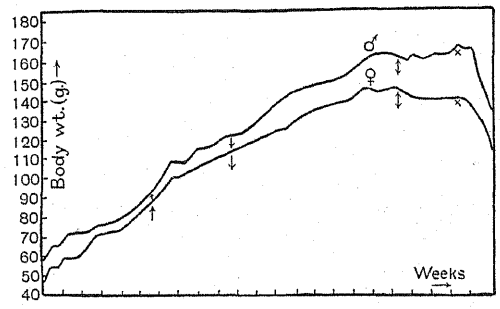


Fig. 9. Dose of vitamin B_1=0·075 cc. of lead filtrate throughout except as stated below; basal diet 14 from beginning of experiment; ↑ diet 26; ↓ diet 30; ↕ diet 44, B_1 supplement stopped; × diet 49 (with no B_1).

kept on a restricted amount of food for 18 days (6 g. per day for the first 3 days and 8 g. daily during the rest of the period). The changes in weight are shown in Table V.

Table V.

Rat No.	Sex	Diet	Period (days)	Initial weight (g.)	Final weight (g.)	Gain in weight in 18 days (g.)
1	♂♀	28	18	155	157	+ 2
2	♂♀	29	18	158	180	+22
3	♂♀	28	18	128	130	+ 2
4	♂♀	29	18	148	172	+24

This clearly shows that the diet containing 40 % lard promotes faster growth under the above conditions than that containing 10 % lard. Lard seems to be in this respect considerably more effective than palm kernel oil or olive oil.

Vitamin B_2, fed in the form of alkaline-autoclaved marmite, does not appear to have any effect [see also Evans and Lepkovsky, 1929].

D. The effect of the ingestion and injection of sodium lactate in vitamin B_1 deficiency.

As the accumulation of lactic acid has been stated to be concerned in the production of both avian and human "beriberi" [Peters, 1930; Hayasaka, 1930] it was considered of interest to investigate the effect of ingested sodium lactate on the rate of decline in weight and on the production of "beriberi" symptoms in vitamin B_1-deficient rats. Experiments were also carried out to observe the effect of the subcutaneous injection of sodium lactate in varying doses into deficient rats.

In this investigation the following diets were used:

Diet 21.	Sucrose	65 parts
	Palm kernel oil	10 ,,
	"Light, white casein"	20 ,,
	Salt mixture (McCollum)	5 ,,
	Cod-liver oil	2·5 ,,
	50 % alkalised marmite	10 cc.

Diet 23 was made up by incorporating 10 cc. of a vitamin B_1 preparation (lead filtrate) per 100 g. of diet 21 so that 10 g. of the diet contained 1·5–2 rat day doses.

Diet 38 was made up by incorporating 10 cc. of 50 % sodium lactate per 100 g. of diet 21.

(*a*) *The ingestion of sodium lactate.*

The method consisted in feeding two groups of adult rats, grown to maturity on whole wheat, brown bread and milk, on diets 21 and 38 respectively and comparing their rates of decline in weight and noting the time of onset of convulsions. Table VI summarises some of the results obtained.

The average rates at which the animals on diets 21 and 38 lost weight under the conditions studied were 8·0 and 12·5 weekly. Although this would tend to indicate a more rapid loss in weight when lactate was present

Table VI.

Rat No.	Sex	Diet No.	Period of feeding on synthetic diet (weeks)	Initial weight (g.)	Final weight (g.)	Total loss in weight (g.)	Loss in weight per week (g.)	Remarks
1	♂	21	6	131	116	15	2·5	Convulsions on 45th day
2	♂	21	6	247	165	82	13·7	Convulsions on 45th day
3	♂	21	6	221	158	63	10·5	Convulsions on 49th day
4	♂	21	6·5	165	148	17	2·6	—
5	♂	21	5·5	135	105	30	5·4	Convulsions on 44th day
6	♂	21	4·5	160	90	70	15·6	Convulsions on 29th day
7	♂	21	3	192	143	49	13·0	Had been on deficient diet for 5 weeks previously; convulsions on 56th day
8	♂	21	8	183	136	47	5·9	Convulsions on 56th day
9	♂	21	11	162	120	42	3·8	Dead on 78th day
10	♂	21	12	207	121	86	7·1	Convulsions on 85th day
11	♂	38	8	172	100	72	9·0	Convulsions on 57th day
12	♂	38	8	193	119	74	9·2	Convulsions on 57th day
13	♂	38	8	222	150	72	9·0	Convulsions on 61st day
14	♂	38	8	240	150	90	11·2	Convulsions on 61st day
15	♂	38	6	187	100	87	14·5	Convulsions on 43rd day
16	♂	38	6·5	178	120	58	9·0	—
17	♂	38	5	188	102	86	17·2	Convulsions on 43rd day
18	♂	38	3	241	178	63	21·0	Had been previously on diet 21; convulsions on 61st day

in the diet, the large individual variations among the members of each group of rats preclude too great a reliance on these figures. It has also to be noted that the presence of lactate in the diet did not hasten the appearance of the "beriberi" convulsions. But here also the values are too scattered to warrant a definite conclusion.

As the metabolism of sodium lactate was likely to be reflected in the hydrogen ion concentration of the urine, the p_H values of the urine of rats on diets 21, 23 and 38, diet 38 + vitamin B_1, and on normal stock diet were measured and are given in Table VII. The urine was collected for 5 hours over toluene.

Table VII.

Rat No.	Sex	Diet	Nature of diet	p_H of urine
1	♀	Normal stock diet	Whole wheat, brown bread and milk	6·5
2	♂	,,	,,	6·5
3	♀	23	+vitamin B_1	6·4
4	♀	23	+ ,,	6·5
5	♂	23	+ ,,	6·5
6	♂	21	− ,,	6·2
7	♂	21	− ,,	6·5
8	♀	21	− ,,	6·4
9	♂	38	−vitamin B_1; +lactate	7·2
10	♂	38	,,	7·2
11	♀	38	,,	7·2
12	♀	38 +vitamin B_1	+vitamin B_1; +lactate	8·6
13	♀	,,	,,	8·6
14	♂	,,	,,	8·7

These results show definitely that while the presence of sodium lactate in the vitamin B_1-deficient diet raises the p_H of the urine from approximately

6·4 to 7·2, the addition of vitamin B_1 to the lactate diet raises the p_H to 8·7. This rise in p_H occurs within 24 hours of the administration of vitamin B_1 to the basal lactate diet.

One rat, weighing 212 g., which was on diet 23, was injected with 2 cc. of 50 % sodium lactate. The p_H values of samples of the urine collected successively after 18 hours and 48 hours were 9·2 and 7·8. The urine collected after 7 days gave the p_H value 6·6, showing a return to the normal.

The significance of these results is discussed later.

(b) The injection of sodium lactate.

The results obtained by the subcutaneous injection of sodium lactate into rats on diets 21 and 23 are summarised in Table VIII. The animals on diet 21 were subjected to a fairly severe deficiency of vitamin B_1, but the deprivation was not sufficiently prolonged to allow them to go into "beriberi" convulsions spontaneously.

Table VIII.

Rat No.	Sex	Diet No.	Nature of diet	Period of feeding on synthetic diet (days)	Body weight at time of injection (g.)	Dose of 50 % sodium lactate injected (cc.)	Results
1	♂	21	− vitamin B_1	45	148	0·2	Survived
2	♀	21	− ,,	52	60	0·4	Survived
3	♀	21	− ,,	54	55	0·4	Died in 4 hours in convulsions
4	♀	21	− ,,	50	90	0·6	Survived
5	♀	21	− ,,	59	70	0·6	Died in an hour
6	♂	21	− ,,	50	95	0·8	Died in 4 hours in convulsions
7	♀	21	− ,,	58	94	0·8	Died in 1½ hours in convulsions; signs of haemorrhage
8	♀	21	− ,,	23	85	0·8	Survived; severe convulsions after 4 days
9	♂	21	− ,,	56	148	1·0	Survived
10	♂	21	− ,,	46	150	1·0	Survived
11	♀	23	+ ,,	59	174	0·8	Survived
12	♀	23	+ ,,	56	105	1·0	Survived
13	♀	23	+ ,,	49	118	1·0	Survived
14	♂	23	+ ,,	49	107	1·6	Died after 5 hours
15	♂	23	+ ,,	61	225	1·6	Survived
16	♂	23	+ ,,	50	142	1·8	Died after 6 hours
17	♀	23	+ ,,	58	122	1·8	Survived
18	♀	23	+ ,,	87	175	2·0	Survived
19	♂	23	+ ,,	49	152	2·0	Died in 3 hours
20	♀	23	+ ,,	56	150	2·0	Survived
21	♂	23	+ ,,	56	185	2·4	Survived
22	♂	23	+ ,,	49	108	2·4	Died in 3 hours
23	♂	23	+ ,,	63	225	2·4	Survived
24	♂	23	+ ,,	64	225	3·2	Convulsions; severe haemorrhage; died in 1 hour

These figures demonstrate that while a dose of about 0·8–1·0 cc. of 50 % sodium lactate per 100 g. body weight is fatal for vitamin B_1-deficient rats, animals receiving vitamin B_1 exhibit a tolerance at least one and a half times as great.

The convulsions observed in the vitamin B_1-deficient animals on injection of sodium lactate sometimes resembled typical "beriberi" convulsions. Attempts were made to find if these artificial convulsions could be cured by the administration of vitamin B_1 preparations, but death was too quick to be prevented by this means.

As the "beriberi" symptoms in pigeons have been ascribed to acidosis produced by lactic acid [Peters, 1930], it was of interest to compare the effects of the injection of similar doses of ammonium chloride, which is known to produce marked acidosis [Salkowski, 1878; Haldane, 1921], into vitamin B_1-deficient and normal rats. The quantities of ammonium chloride injected were approximately equimolecular to some of the smaller doses of sodium lactate used in the preceding set of experiments. Ammonium chloride was, however, found to be much more toxic than sodium lactate and appeared to be equally fatal to normal and deficient rats, in the doses tried (Table IX).

Table IX.

Rat No.	Sex	Diet	Period on synthetic diet (days)	Body weight at time of injection (g.)	Dose of 22·2 % sol. of NH_4Cl injected (cc.)	Results
1	♂	21	56	90	0·9	Died in 15 minutes
2	♂	21	49	80	0·45	Died in 15 minutes
3	♀	21	—	100	0·9	Died in 10 minutes
4	♀	21	—	147	0·9	Died in 15 minutes
5	♂	23	56	198	1·8	Died in 5 minutes (severe haemorrhage)
6	♂	23	56	200	0·9	Died in 20 minutes
7	♀	23	57	170	0·9	Died in 15 minutes

E. The oxidative mechanism of tissues in vitamin B_1-deficiency.

Suggestions regarding the impairment of the oxidative apparatus in "vitamin B" deficiency are numerous. Drummond and Marrian [1926, where the literature up to 1926 is quoted], from a series of experiments carried out with rats, found, however, no experimental support for the theory. The question has been very recently revived by Abderhalden and Vlassopoulos [1931] working with the liver, muscle and brain tissues of pigeons.

The present work was undertaken in view of the statement of Peters [1930] regarding an increase in the production of lactic acid in vitamin B_1 deficiency in the pigeon and its concentration in the brain, and of the recent experiments of Roche [1931], which also tend to show an accumulation of three-carbon bodies in the blood of avitaminous pigeons. It was considered, firstly, that if in vitamin B_1 deficiency the specific mechanism concerned in the removal of lactic acid was involved, its impairment might not be revealed by the technique employed by Drummond and Marrian, who used succinate as the metabolite in their anaerobic reduction experiments with methylene blue. The succino-dehydrogenase system might be unimpaired in vitamin B_1 deficiency, while the lactic dehydrogenase system might be damaged. Secondly, the experiments

on the direct oxygen uptake of tissues might not lead to any definite conclusions on this question, because the lactic-oxidising system, while being qualitatively important, might not be quantitatively so great in relation to the total uptake that its impairment would cause a markedly diminished oxygen consumption. This diminution would have to be sufficiently large to be outside the error caused by the fortuitous presence of various metabolites in the particular tissue examined at the moment of killing the animal. As in such experiments the oxygen uptakes of the tissues of different animals have to be compared, the variation introduced by this factor is likely to be large. A more correct idea of the condition of the lactic-oxidising system would be obtained by measuring the increased oxygen uptake due to added lactate, though there might not be a strictly proportional relation between the two owing to the competition of different substrates being involved in the question. Thirdly, since Kinnersley and Peters [1930] have shown that lactic acid accumulates chiefly in the brain, it was considered possible that while other "beriberi" tissues might not show any marked difference in oxygen consumption from normal tissues, the brain might reveal the difference. The liver might also be concerned in this question, as it apparently plays an important part in lactic acid metabolism [Cori and Cori, 1929].

In the following experiments, therefore, which were carried out throughout with the brain and liver tissues of rats, the significant values are considered to be those that were obtained after the addition of sodium lactate as substrate, both in the anaerobic experiments with methylene blue and the experiments on direct oxygen consumption.

(1) *Experiments with Thunberg's methylene blue technique.*

The Thunberg tubes were arranged according to the following general scheme.

	Tubes without lactate cc.	Tubes with lactate cc.
Phosphate buffer (p_H 7·4)	2·0	2·0
Methylene blue (1/5000)	1·0	1·0
Na lactate (M/60)	—	0·5
Distilled water	2·0	1·5
Tissue suspension in phosphate buffer	1·0	1·0

Temp. of bath 37°.

The results of certain typical experiments with liver and brain tissues of normal and vitamin B_1-deficient animals are shown in Table X.

Figures of a similar order were obtained in other experiments. Thus, with 0·148 g. of the brain tissue of one deficient rat the time of reduction of methylene blue in presence of sodium lactate was 4·1 minutes, the reduction time without lactate being 18·2 minutes, showing a fair degree of efficiency of the mechanism concerned in the oxidation of lactic acid.

Though both the liver and brain tissues of normal animals reduce methylene blue more rapidly than the corresponding tissues of vitamin B_1-deficient

Table X.

Liver tissue.

		Time of decoloration without lactate (mins.)		Time of decoloration with lactate (mins.)	
Unwashed liver tissue (0·15 g.)	Normal	5·5 5·75 5·7	5·65	5·0 4·05 4·15	4·4
	Vitamin B_1-deficient	11·6 9·25 10·0	10·28	6·6 7·2 7·2	7·0
Washed liver tissue* (0·15 g.)	Normal	Slight decoloration in 3 hours		35·2 40·25	37·72
	Vitamin B_1-deficient	,,		49·0 46·0	47·5
	Brain tissue.				
Washed brain tissue† (0·14 g.)	Normal	26·1 24·0 24·4	24·8	6·1 5·6 5·9	5·87
	Vitamin B_1-deficient	37·25 38·0 37·5	37·58	7·0 6·9 6·8	6·9

* Washed twice with 100 cc. of Ringer-Locke solution per 1·5 g. of tissue. Too much washing apparently causes a large loss of lactic dehydrogenase.

† Washed once with 100 cc. Ringer-Locke solution per 1·4 g. tissue.

animals in the absence of sodium lactate (Table X), no valid conclusion regarding the oxidative mechanism can be drawn from these figures, as has been pointed out earlier. More significant values are obtained by calculating the ratio $(a:b)$ of the time of reduction of methylene blue without lactate to the time of reduction with lactate in each case (Table XI).

Table XI.

	$a:b$
Liver tissue of normal rat	1·30
,, deficient rat	1·47
Brain tissue of normal rat	4·22
,, deficient rat	5·44
,, ,,	4·50

These ratios indicate that the acceleration of the reduction of methylene blue due to added lactate by normal and deficient liver and brain tissues is approximately of the same order for each type of tissue. The variations fall within the limits of error of the technique.

(2) *Experiments on the oxygen consumption with the Barcroft apparatus.*

These experiments were carried out with the chopped whole brain tissue (unwashed).

The general arrangement of the cups is shown in Table XII. Temp. 38°.

Table XII.

	Without lactate		With lactate	
	Left-hand cup cc.	Right-hand cup cc.	Left-hand cup cc.	Right-hand cup cc.
Phosphate buffer (p_H 7·2)	1·0	1·0	1·0	1·0
Sodium lactate ($M/10$)	—	—	—	0·5
Ringer solution	1·5	0·5	1·5	0·5
Distilled water	0·5	0·5	0·5	—
Brain tissue suspension in phosphate buffer	—	1·0	—	1·0

Some results are shown in Figs. 10 and 11 which represent extreme values. Thus in one case the rate of oxygen consumption of the normal tissue was

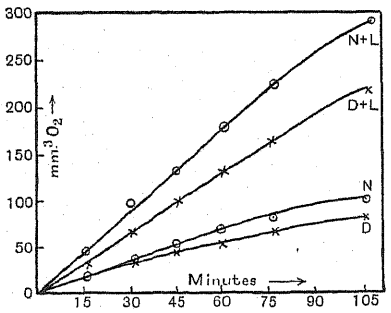


Fig. 10.

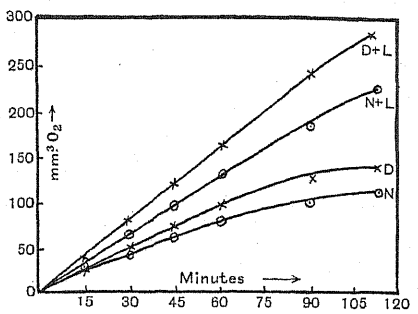


Fig. 11.

Fig. 10. Oxygen consumption of 0·1 g. brain tissue.
× — × — × Tissue of deficient animal in convulsions (D).
⊙ — ⊙ — ⊙ Tissue of normal animal (N).
D + L stands for "beriberi" tissue with added lactate.
N + L stands for normal tissue with added lactate.

Fig. 11. Oxygen consumption of 0·13 g. brain tissue.
× — × — × Tissue of deficient animal (D).
⊙ — ⊙ — ⊙ Tissue of normal animal (N).
D + L stands for "beriberi" tissue with added lactate.
N + L stands for normal tissue with added lactate.

somewhat greater than that of the "beriberi" tissue, while in the other case the situation was reversed, though this latter case was somewhat exceptional.

Addition of lactate as substrate resulted in a very marked increase in the rate of oxygen uptake of both the normal and "beriberi" tissues. The orders of increase, however, show some variations, and it appears that this technique would not reveal any but a considerable damage to the lactic-oxidising mechanism in vitamin B_1 deficiency, unless a statistical analysis of a very large number of results were made.

The cytochrome content of normal and "beriberi" tissues.

The brain, liver, kidney and heart muscle tissues of normal rats and vitamin B_1-deficient rats in convulsions (which were nearly killed by chloroform and then bled to death) were thoroughly washed and examined for the cytochrome bands in the usual way with the microspectroscope, after the addition

of a trace of sodium hydrosulphite, if necessary. All the cytochrome bands were equally prominent in the corresponding tissues of normal and avitaminous animals. There was, apparently, no diminution of the cytochrome content in vitamin B_1 deficiency.

Discussion.

The experiments described in Section A offer no support to the contention of Randoin and Simonnet [1924] that the requirement of vitamin B_1 has a relation to the carbohydrate content of the diet of pigeons. On the other hand, it has to be pointed out that these experiments on the relation between vitamin B_1 and the protein/carbohydrate ratio of the diet constitute no evidence against the theory that vitamin B_1 plays some part in carbohydrate metabolism, as it is quite possible that the vitamin is required also for the degradation of the glucogenic residue of the protein molecule. The non-dependence of the requirement of vitamin B_1 on the nature of the dietary carbohydrate (glucose, fructose, sucrose and maltose) is not probably of much significance, because presumably all these carbohydrates yield the same three-carbon compounds by degradation and, if vitamin B_1 is concerned in the metabolism of the latter [Peters, 1930; Vogt-Møller, 1931; Roche, 1931] it is quite reasonable that the requirement of vitamin B_1 should be independent of the nature of the carbohydrate. The decline in weight of animals receiving galactose as the sole source of carbohydrate is probably to be related to its inefficient utilisation and its low renal threshold. Its absorption through the intestinal wall is known to be rapid [Cori, 1925; McCance and Madders, 1930]. The lactose diet had a somewhat deleterious effect in that it tended to produce diarrhoea.

The experiments on the relation between vitamin B_1 and dietary fat indicate that all fats are not of equal value so far as the sparing action on vitamin B_1 is concerned. Thus lard is apparently more efficient in this respect than palm kernel oil and olive oil. It is, therefore, not clear how far the sparing action is the effect of fat *per se*. While the observations of Evans and Lepkovsky [1929] on this subject might be interpreted to mean that the vitamin plays some rôle in carbohydrate metabolism, other possible explanations might also be suggested. Thus, assuming that the vitamin acts only by stimulating the appetite in some unknown way, it is possible that in Evans and Lepkovsky's experiments, if the limiting factor in the growth of the deficient rats were calorie intake, the rats receiving fat-rich diets would grow better because of the relatively much greater calorie intake. The observation mentioned in this paper, that rats receiving restricted amounts of a diet containing 40 % lard grew better than those receiving the same quantities of a diet containing 10 % lard when both groups were given a sub-optimum dose of vitamin B_1, might also be interpreted in a similar manner. The phenomenon of refection reminds one also of a possible function of the intestinal bacteria of rats subsisting on high-fat diets. It may be mentioned incidentally that the high-fat diets did not exert any deleterious effect on the animals [see also Osborne

and Mendel, 1924], except that they were greasy looking and somewhat sluggish.

As described in Section D the appearance of symptoms was not markedly hastened by the presence of sodium lactate in a vitamin B_1-deficient diet. The absorption of sodium lactate is known to be fairly rapid [Cori, 1930] and the change in p_H of the urine indicates that the ingested sodium lactate was being metabolised. The expectation that sodium lactate might precipitate the appearance of symptoms in deficient rats was based on the observation of Kinnersley and Peters [1930] regarding the accumulation of lactic acid in the brain and on the evidence presented by McGinty [1929] to show that brain tissue normally removes some of the lactic acid from circulation. The p_H (8·4) of the urine of rats receiving the lactate diet *plus* vitamin B_1, which was higher than the p_H (7·2) of the urine of rats receiving the lactate diet *minus* vitamin B_1, might be interpreted to indicate a greater efficiency of the animals receiving vitamin B_1 in metabolising ingested lactate. This phenomenon might, however, also be ascribed to the diminished consumption of the lactate diet in vitamin B_1 deficiency. How far the ability to dispose of lactic acid is affected in vitamin B_1 deficiency is at present under investigation in experiments both *in vivo* and *in vitro*.

The injection experiments with sodium lactate showed a definitely decreased tolerance to lactate in vitamin B_1 deficiency. It is possible, however, that this was due to the comparatively weak condition of the deficient rats. The convulsions induced by the injection of lactate sometimes resembled "beriberi" convulsions, but insulin convulsions are also similar in appearance.

The significant evidence brought forward by Kinnersley and Peters [1929, 1930] regarding an increase in blood-lactic acid and a localised lactic acidosis in the brain finds support in the recent experiments of Roche [1931] on the blood of deficient pigeons, and of Hayasaka [1930], who observed that sodium lactate, injected intravenously into normal and cured "beriberi" human subjects, disappeared faster than that injected into "beriberi" subjects. As has been indicated earlier, the evidence for the depressed oxidation of various tissues of deficient pigeons [Abderhalden and Vlassopoulos, 1931], and for the diminished respiration of the blood of "beriberi" subjects [Kimura, 1930], might be misleading unless the differences are of a relatively high order, and unless a very large number of measurements are taken. In a recent note, which appeared after the present work had been completed, Gavrilescu and Peters [1931] have recorded the interesting observation that the lowering of the oxygen consumption of the brain tissue of avitaminous pigeons was confined especially to certain parts of the brain. If such be the case, it might have escaped detection in the experiments described in Section E, which were carried out with the whole brain tissue of rats. These experiments on the increased oxidation due to added lactate indicate, however, that the mechanism concerned in the oxidative removal of the particular metabolite, lactate, is not seriously damaged in vitamin B_1 deficiency. While it is possible that a

statistical analysis of a large number of measurements might indicate a small amount of injury to this mechanism, it is considered probable that, so far as the lactic acid observed to accumulate by Kinnersley and Peters is concerned, it is the mechanism of its "synthetic" removal which is damaged rather than that of its oxidative removal. Holmes and Ashford [1930] have demonstrated the existence of a Meyerhof quotient for the brain, though at the moment no information is available as to the fate of the lactic acid which is removed but not oxidised by brain tissue. In fact, our knowledge about the steps by which the lactic acid in muscle is resynthesised into glycogen, is also very meagre [Long and Grant, 1930]. Investigation is in progress to test the accuracy of the above suggestion.

SUMMARY.

1. The requirement of vitamin B_1 for young growing rats, receiving a sub-optimum dose of vitamin B_1, was found to be independent of the protein/carbohydrate ratio of the diet.

2. The requirement of vitamin B_1 under the same conditions was likewise independent of the nature of carbohydrate in the diet, glucose, fructose, sucrose or maltose. With galactose and lactose as sole sources of carbohydrate in the diet the animals were found to decline in weight.

3. The relation of the requirement of vitamin B_1 to the dietary fat has been investigated. While palm kernel oil and olive oil had little sparing action on the vitamin, lard appeared to have a definite effect.

4. The ingestion of sodium lactate did not appreciably hasten the appearance of symptoms in vitamin B_1-deficient rats. The p_H of the urine of animals receiving lactate *plus* vitamin B_1 was higher than that of animals receiving lactate *minus* vitamin B_1. The lethal dose of injected sodium lactate was lower for deficient animals than for normal ones.

5. The cytochrome content of the liver, kidney, brain and heart muscle tissues of rats was not diminished in vitamin B_1 deficiency. The mechanism for the oxidation of lactic acid by the liver and brain tissues of deficient rats appeared to remain fairly efficient.

I am indebted to Sir F. G. Hopkins for his interest in this work and to Dr Keilin and Dr Cook for generous help at certain stages of this investigation. To Miss R. Leader my thanks are due for able assistance with the animals.

REFERENCES.

Abderhalden and Vlassopoulos (1931). *Pflüger's Arch.* **226**, 808.
Campbell and Soskin (1928). *J. Clin. Invest.* **6**, 291.
—— and Maltby (1928). *J. Clin. Invest.* **6**, 303.
Cori (1925). *J. Biol. Chem.* **66**, 691.
—— (1930). *J. Biol. Chem.* **87**, 13.
—— and Cori (1929). *J. Biol. Chem.* **81**, 389.
Drummond and Marrian (1926). *Biochem. J.* **20**, 1229.

Eijkman (1897, 1). *Virchow's Arch.* **148**, 523.
—— (1897, 2). *Virchow's Arch.* **149**, 187.
Evans and Lepkovsky (1929). *J. Biol. Chem.* **83**, 269.
Funk (1914). *Z. physiol. Chem.* **89**, 378.
Gavrilescu and Peters (1931). *Chem. Ind.* **50**, 442; *Biochem. J.* **25**, 1397.
Grijns (1901). *Arch. Schiff. Trop.-Hyg.* **5**, 302.
—— (1907). *Arch. Hyg.* **62**, 128.
Guha (1931, 1). *Biochem. J.* **25**, 931.
—— (1931, 2) *Biochem. J*, **25**, 1385.
Haldane (1921). *J. Physiol.* **55**, 265.
Hartwell (1925). *Biochem. J.* **19**, 1075.
Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
Hayasaka (1930). *Tohoku J. Exp. Med.* **14**, 283.
Holmes and Ashford (1930). *Biochem. J.* **24**, 1119.
Kimura (1930). *Tohoku J. Exp. Med.* **15**, 112.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
—— —— (1930). *Biochem. J.* **24**, 711.
Long and Grant (1930). *J. Biol. Chem.* **89**, 553.
McCance and Madders (1930). *Biochem. J.* **24**, 795.
McGinty (1929). *Amer. J. Physiol.* **88**, 312.
Osborne and Mendel (1924). *J. Biol. Chem.* **59**, 13.
Peters (1930). Harben Lectures, *J. State Med.* **38**, No. 2.
Randoin and Simonnet (1924). *Bull. Soc. Chim. Biol.* **6**, 601.
Reader and Drummond (1926). *Biochem. J.* **20**, 1256.
Roche (1931). *Bull. Soc. Chim. Biol.* **13**, 186.
Rose, Giragossintz and Kirstein (1929–30). *Proc. Soc. Exp. Biol. Med.* **27**, 523.
Salkowski (1878). *Z. physiol. Chem.* **1**, 1.
Vogt-Møller (1931). *Biochem. J.* **25**, 418.

CL. ON GALACTOSE AS THE DIETARY CARBOHYDRATE.

By BIRES CHANDRA GUHA[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 3rd, 1931.*)

In course of an investigation on a possible relationship between the requirement of vitamin B_1 and the nature of the dietary carbohydrate [Guha, 1931] it was observed that if galactose formed the sole carbohydrate in a fat-free diet the rats rapidly declined in weight and died. When young rats of about 50 g. in weight were used, death occurred within 3–4 days. Uniform results were obtained with galactose (extra pure) of the British Drug Houses and with Kahlbaum's product. Though the utilisability of galactose is known to be relatively poor, this unique behaviour of galactose among the carbohydrates studied merited further attention, especially as such studies might have some relation to the still unsolved problem of the significance of the occurrence of lactose in milk. In the present investigation the effect of a galactose diet unsupplemented by any other adequate source of non-protein energy on young and adult rats has been studied. Experiments have also been carried out to find how far the ill-effects observed could be averted by the presence of varying amounts of glucose, fat and protein in the diet. The behaviour of mice under similar circumstances has also been studied in a few preliminary experiments. The results of the work, though incomplete, are published at this stage as the investigation cannot be pursued further at the moment.

Experimental.

The rats used were mostly of the black and white strain, a few being albinos. For comparative experiments litter-mates were employed. As usual, the animals were kept in separate cages and all vitamin supplements were fed separately from the basal diet except when yeast was used. The vitamin B_1 and B_2 preparations used were the same as employed in the work described in the preceding communication [Guha, 1931]. Each animal received 2 drops of cod-liver oil daily. In several cases records of the food and water intake and of the urinary output were kept.

The following diets were used. The caseinogen used was the "light, white" variety of the British Drug Houses.

[1] Travelling Fellow of the University of Calcutta.

Diet 19	Parts
Galactose	75
Caseinogen	21
Salt mixture (McCollum)	4

Diet 19 (*a*)	Parts
Galactose	37·5
Glucose	37·5
Caseinogen	21
Salt mixture	4

Diet 19 (*b*)	Parts
Galactose	37·5
Palm kernel oil	37·5
Caseinogen	21
Salt mixture	4

Diet 19 (*c*)	
Galactose	70
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*d*)	
Galactose	35
Glucose	35
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*f*)	
Glucose	70
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*g*)	
Galactose	60
Palm kernel oil	16
Caseinogen	21
Salt mixture	4

Diet 19 (*h*)	
Galactose	35
Caseinogen	56
Dried yeast	5
Salt mixture	4

Diet 19 (*j*)	
Galactose	70
Caseinogen	19
Sodium dihydrogen phosphate	2
Dried yeast	5
Salt mixture	4

Diet 19 (*k*)	
Galactose	70
Palm kernel oil	10
Caseinogen	21
Salt mixture	4

Diet 19 (*l*)	
Galactose	70
Palm kernel oil	5
Caseinogen	21
Salt mixture	4

Diet 1	
Glucose	75
Caseinogen	21
Salt mixture	4

A. *Experiments with adult rats.*

Rats which had been growing for a considerable period on diet 1, containing glucose supplemented by vitamin B_1 and B_2 preparations and cod-liver oil, were fed on diet 19 with the same supplements. The rats rapidly declined and died (Fig. 1).

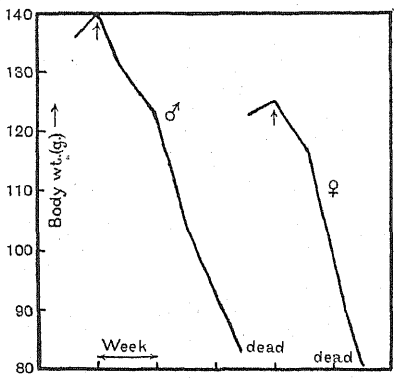


Fig. 1. ↑ Diet 19 supplemented with vitamin B_1 and B_2 preparations.

B. *Experiments with young rats.*

Litters of young rats were broken up into groups of two. The behaviour of these groups on diets 19 (*c*) containing galactose, 19 (*f*) containing glucose, 19 (*d*) containing equal parts of glucose and galactose, 19 (*j*) containing galactose with extra phosphate and 19 (*h*) containing galactose with extra caseinogen

is illustrated in Figs. 2 and 3. All the diets contained 5 % yeast as the source of "vitamin B," which contributed to a small extent to the energy value of

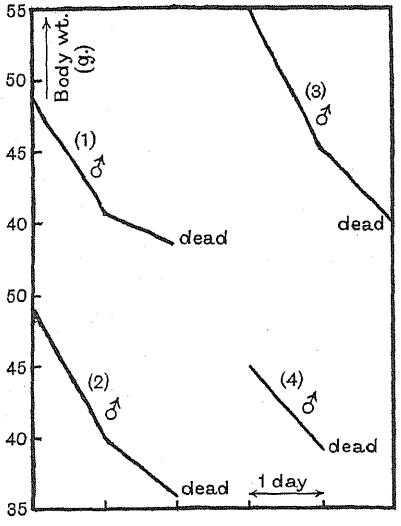


Fig. 2.

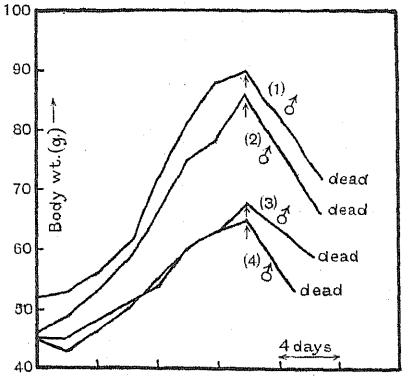


Fig. 3.

Fig. 2. Curve (1). Diet 19 (*c*). Curve (2). Diet 19 (*c*). Curve (3). Diet 19 (*h*). Curve (4). Diet 19 (*h*).

Fig. 3. Curve (1). Diet 19 (*f*) from beginning of experiment; ↑ diet 19 (*c*).
Curve (2). Diet 19 (*f*) from beginning of experiment; ↑ diet 19 (*c*).
Curve (3). Diet 19 (*d*) from beginning of experiment; ↑ diet 19 (*j*).
Curve (4). Diet 19 (*d*) from beginning of experiment; ↑ diet 19 (*j*).

the diets. In diet 19 (*j*) extra phosphate was incorporated to find if it had any influence on the utilisation of galactose. Diets 19 (*c*), 19 (*j*) and 19 (*h*) were unable to support life, while the animals receiving diet 19 (*d*) grew at a markedly slower rate than those receiving diet 19 (*f*). This last case shows that 35 % glucose cannot support the normal growth of young rats and that the same quantity of galactose cannot replace it in the diet.

(*a*) *Food and water intake.* In Table I are given the figures for the food and water intakes of some animals receiving diets 19 (*f*) and 19 (*d*). Although the animals receiving diet 19 (*d*) were growing more slowly than those receiving diet 19 (*f*), the former were actually consuming more food than the latter. The water intake was also considerably higher.

Table I.

Rat No.	Sex	Diet	Period over which food intake records were kept (days)	Average daily food intake (g.)	Average daily water intake (cc.)
1	♂	19 (*f*)	10	9·9	22·6
2	♂	19 (*f*)	15	10·6	17·8
3	♂	19 (*d*)	10	12·6	31·2
4	♂	19 (*d*)	15	12·8	34·8

The animals receiving diet 19 (*c*) consumed larger quantities of food and took much more water than those of any other group. It was difficult, however, to keep accurate food intake records owing to the scattering of the diet. The urinary output of these animals was also considerably higher, reaching as much as 40–45 cc. daily.

(*b*) *Condition of the animals.* The rats on diet 19 (*c*) soon lost the lustre of the eye and became hunched. The fur became ruffled and other signs of weakness resembling those observed in vitamin B_1-deficient rats were also present. The animals lay sometimes in a comatose condition with extended limbs for 20 hours before death. In this condition there was no appreciable response to two injections of a total volume of 1·2 cc. of 10 % glucose.

Post mortem examination, kindly carried out by Mr J. R. M. Innes, revealed no organic lesions. The intestinal tract had an unhealthy appearance.

C. *Experiments with young rats.*

In Fig. 4 are illustrated the growth curves of young rats on diets 1, 19, 19 (*a*), 19 (*b*), 19 (*g*), 19 (*k*), 19 (*l*) and 19 (*c*). Of these diets all except diet

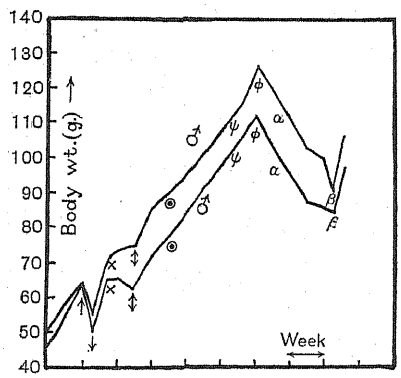


Fig. 4.

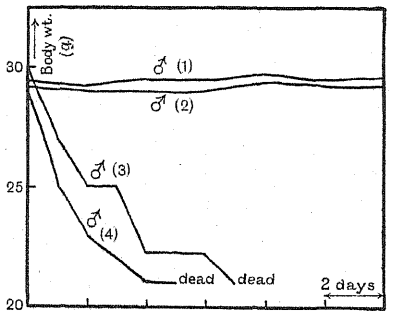


Fig. 5.

Fig. 4. Diet 1 from beginning of experiment; ↑ diet 19; ↓ diet I; × diet 19 (*a*); ↕ diet 19 (*b*); ⊙ diet 19 (*g*); ψ diet 19 (*k*); φ diet 19 (*l*); α diet 19 (*c*); β "complete" synthetic diet.

Fig. 5. Experiments with mice. Curve (1). Diet 19 (*f*). Curve (2). Diet 19 (*f*). Curve (3). Diet 19 (*c*). Curve (4). Diet 19 (*c*).

19 (*c*), which contained yeast, were supplemented daily by 1 cc. of a vitamin B_1 concentrate and 1 cc. of a vitamin B_2 preparation, so as to eliminate as far as possible the source of energy provided by the glycogen, fat and protein of yeast. Diet 19 (*g*) was made up so as to contain an amount of palm kernel oil approximately isodynamic with 37·5 g. glucose. This was done in order to compare the effects of this diet and of diet 19 (*a*) which are calorifically approximately equivalent, neglecting galactose as a source of energy. It will be observed that diet 19 (*g*) has a distinctly better effect. It will also be noticed that, by gradually lowering the content of fat in the galactose diet, growth can be progressively inhibited. (Compare diets 19 (*b*), 19 (*g*), 19 (*k*)

and 19 (*l*).) Food intake records were kept throughout this set of experiments, which show that the greatest food consumption occurred with diet 19 (*c*), approaching sometimes 23 g. daily.

D. *Experiments with mice.*

Only a few preliminary experiments could be carried out with mice[1]. These have yielded results similar to those obtained with rats. These animals, which were nearly adult, were fed on diets 19 (*f*) and 19 (*c*). The results are illustrated in Fig. 5. It will be observed that, while the glucose diet was apparently adequate, the galactose diet failed to support life.

The striking fact about the mice receiving diet 19 (*c*) was that they exhibited all the symptoms typical of "beriberi" in the rat in addition to those observed in the rats on the same diet. Thus, after 5–7 days on this diet there was loss of muscular co-ordination and they went into convulsions when swung, which were indistinguishable from "beriberi" convulsions in the rat. The animals could remain in the moribund condition for over 40 hours. It has to be mentioned that they were eating considerable quantities of food for the first three days and were, therefore, receiving ample quantities of yeast from the diet.

Discussion.

The results described indicate that the degree to which galactose can be utilised by the rat is singularly low. Death can be prevented by replacing part of the galactose by glucose or by fat but not by increasing the protein content of the diet. That the limiting factor in the galactose diet is its inability to supply sufficient energy to the rat is also indicated by the proportionate growth responses to graded amounts of fat in the galactose diet. The increased water intake and diuresis on the galactose diet is readily intelligible in the light of our knowledge about the rapid absorption of galactose through the intestinal wall [Cori, 1925; McCance and Madders, 1930] and of its low renal threshold [Folin and Berglund, 1922]. In fact, this appears to be the reason why death is so quick in young animals on the galactose diet. As has been pointed out, the food intake on this diet is greater than on any other, which indicates that the low utilisability of galactose prompts the consumption of more food and, as galactose is quickly absorbed and cannot be readily oxidised or converted into glycogen [Lusk, 1915; Weinland, 1899; Ishimori, 1912–13], it has to be excreted. Thus, the whole process of digestion, absorption and excretion involves a greater strain than would have been the case if the animal were only starving and, therefore, apparently precipitates death. In this matter these animals behave differently from vitamin B_1-deficient rats, because, while the former continue to consume food till shortly before death and undergo virtual but not actual starvation, the vitamin B_1-deficient animals, on the other hand, starve themselves voluntarily. Attention has to

[1] I am indebted to Miss E. Turner for supplying me with these animals.

be drawn to the fact that many of the symptoms are common to both groups of animals, which, again, supports the contention that in vitamin B_1 deficiency the usual symptoms are simply those of starvation, consequent on anorexia. The typical symptoms of "beriberi" observed in mice subsisting on the galactose diet and receiving ample "vitamin B" also indicate the non-specificity of the so-called "beriberi" symptoms.

These experiments make the question of the function of lactose in nutrition still more puzzling. While Folin and Berglund [1922] have shown that the utilisation of galactose is apparently increased in presence of glucose, the present investigation indicates the desirability of the study of the rôle of fat in this connection. It appears from this study that the energy value of the fat in milk might be nearly as important for the nutrition of the young as are the vitamins it carries.

SUMMARY.

Young and adult rats on a fat-free diet containing galactose as the sole carbohydrate rapidly decline in weight and die. The symptoms of these animals are described. Mice behave similarly under the same conditions and develop typical "beriberi" symptoms although receiving ample quantities of "vitamin B." How far growth can be supported in young rats by incorporating varying quantities of glucose, fat and protein in the galactose diet has been studied.

My thanks are due to Sir F. G. Hopkins for criticism and advice.

REFERENCES.

Cori (1925). *J. Biol. Chem.* **66**, 691.
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 213.
Guha (1931). *Biochem. J.* **25**, 1367.
Ishimori (1912–13). *Biochem. Z.* **48**, 332.
Lusk (1915). *J. Biol. Chem.* **20**, 555.
McCance and Madders (1930). *Biochem. J.* **24**, 795.
Weinland (1899). *Z. Biol.* **38**, 16.

CLI. THE CHEMICAL NATURE OF VITAMIN B_1 FROM EVIDENCE AFFORDED BY ITS ELECTRICAL TRANSFERENCE.

BY THOMAS WILLIAM BIRCH AND BIRES CHANDRA GUHA[1].

From the Nutritional Laboratory and the Biochemical Laboratory, Cambridge.

(*Received July 2nd, 1931.*)

SINCE the recognition of the dietary principle now known as vitamin B_1 a great number of attempts have been made to isolate it during the last thirty years. Though nearly all these attempts have admittedly failed in their main objective, the methods of fractionation generally employed suggest that the vitamin is probably of a basic nature. This conclusion is, however, complicated by the probability that adsorption plays a more important rôle in these fractionations than does true chemical reaction. Moreover, ample evidence has been brought forward by several investigators [Kinnersley and Peters, 1928; Guha and Drummond, 1929] to show that the behaviour of vitamin B_1 is to a large extent modified by the associated substances present. The adsorption of vitamin B_1 by fuller's earth and "acid clay," however, is apparently of the polar type and, therefore, strengthens the supposition that vitamin B_1 is a base. All this evidence, however, is indirect. The present paper records the results of experiments on the behaviour of various preparations of vitamin B_1, as also of crude yeast extracts, on being subjected to electrodialysis at different hydrogen ion concentrations. This investigation had three objects in view:

(1) to obtain what might be regarded as direct and conclusive evidence as to the basic nature of vitamin B_1 and to attempt to find the approximate p_K value of its basic group;

(2) to examine the possibilities of concentration of vitamin B_1 by this method;

(3) to attempt a separation of vitamin B_1 from other members of the vitamin B complex.

After this work had been completed, our attention was drawn to a paper by Williams and Waterman [1929], in which they discuss the possibility of the separation of ampholytes by electrodialysis using a multiple compartment cell. They state that "the two factors which can be recognised most definitely by our present feeding technique migrate towards the alkaline region where they undergo fairly rapid decomposition. These factors are the antineuritic

[1] Travelling Fellow of the University of Calcutta.

vitamin and the one designated as 'the third factor.'" No details, however, are given. Professor Drummond has also recently informed us of the unpublished work of A. F. Watson in his laboratory in 1921, who observed with crude yeast extracts that "vitamin B" (its complex nature not being established at that time) migrated towards the cathode at p_H 3·8.

Experimental.

A three compartment cell was used for the electrolysis. It was made by cutting a wooden box, open at the top and of suitable dimensions, into three vertical sections of equal length. Each section was soaked in paraffin-wax to render it impermeable to water. Four rubber washers were cut to fit the ends of the sections, a parchment membrane was placed between each pair so as to separate the three sections, and the whole box was then clamped together by means of two iron clamps. The vitamin solution was placed in the middle compartment, the two end compartments each being filled with distilled water in which platinum electrodes were immersed. The cathode electrode acted both as stirrer and electrode. A cork pulley coupled to an electric motor was fitted to the upper end of a glass tube, the other end being fused on to the platinum and connection made by means of a copper wire dipping into mercury contained in the tube. This procedure was found necessary as in a previous experiment, using stationary carbon electrodes, a nearly total loss of activity was recorded. This was thought to be due either to adsorption on the carbon or to a local concentration of alkali near the electrode inactivating the vitamin. A cooling coil made of glass tubing was placed in each compartment. The coils were connected in series with a pump and a coil of lead tubing immersed in a freezing mixture, brine being continuously pumped round through this closed circuit to conduct away the heat generated by the passage of the current. During the course of the electrolysis sulphuric acid was added to the cathode compartment and baryta to the anode. The p_H of the middle compartment was similarly adjusted when necessary. In some of the experiments, as indicated below, certain substances were introduced into the end compartments, both to act as buffers and to reduce the resistance of the cell. The p_H was determined colorimetrically by the use of external indicators.

Exp. 1. Electrolysis of lead filtrate at p_H *7 and* p_H *4.*

50 cc. of a lead filtrate fraction prepared by extracting yeast with hot 50 % alcohol, precipitating with lead acetate and removing the lead with hydrogen sulphide were diluted to 150 cc., placed in the middle compartment and adjusted to p_H 7 with baryta. 150 cc. of distilled water were placed in both the anode and cathode chambers. The cathode liquid was kept at p_H 1·5–2, the anode liquid at p_H 2–5. Electrolysis was carried on for 4 hours with a current of 0·8 amp. (the temperature being 10–15°). At the end of this time 15 cc. or 1/10 of the total volume of the middle compartment and

the whole of the anode and cathode solutions were removed for feeding tests. The barium was precipitated by sulphuric acid, the precipitate filtered and washed, and the filtrates and washings were made up to definite volumes. The dosage was computed by finding the number of day-doses present after electrolysis on the basis of the original activity present in the middle compartment. The method of assay of the vitamin was essentially the same as that employed by Guha [1931]. Fig. 1 shows the activity of these solutions and also that

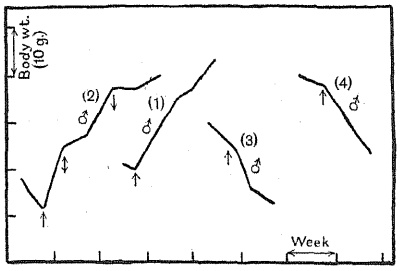


Fig. 1.

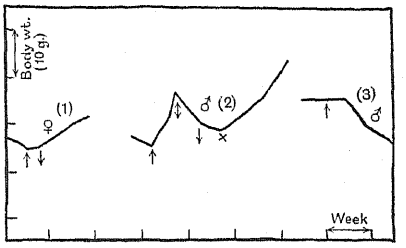


Fig. 2.

Fig. 1.

Curve (1). Lead filtrate before electrolysis; ↑ 0·15 cc. (day-dose).
Curve (2). Fraction of middle chamber after electrolysis at p_H 7·0; ↑ dose≡3 times day-dose of original lead filtrate; ↕ dose≡1·5 times day-dose of original lead filtrate; ↓ dose≡1 original day-dose.
Curve (3). Cathode fraction; ↑ dose≡3 times original day-dose.
Curve (4). Anode fraction; ↑ dose≡6 times original day-dose.

Fig. 2. Electrolysis at p_H 4·4 (Exp. 1).

Curve (1). Middle fraction; ↑ dose≡1·5 times original day-dose; ↓ dose≡4·5 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡1·8 times original day-dose; ↕ dose≡1 original day-dose; ↓ dose≡1·5 times original day-dose; × dose≡2 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡9 times original day-dose.

of the original lead filtrate. The greater part of the vitamin is seen still to be contained in the middle compartment, the cathode and anode fractions showing no activity. The fluid from the middle compartment was then adjusted to p_H 4·4 and further electrolysed for 4½ hours the current being 0·3 amp. From Fig. 2 it is seen that over 50 % of the activity has passed into the cathode compartment while the middle compartment contains the remainder of the activity, the anode fraction being totally inactive.

Exp. 2. Re-electrolysis of cathode solution at p_H *8·5.*

80 cc. of the active cathode solution obtained at the previous stage were adjusted to p_H 8·5 by means of baryta and re-electrolysed for half an hour with a current of 0·3 amp. at 10–15°. Attempts were made to keep the anode and cathode liquids at approximately p_H 8·5 by buffering them with small amounts, about 0·3 g., of glycine and ammonium sulphate respectively. Owing to the rapid liberation of acid and alkali in these compartments great difficulty was experienced, but for the most part they were kept on the alkaline side of

neutrality. Fig. 3 shows that about 50 % of the vitamin has migrated to the cathode and none to the anode, which confirms the earlier finding.

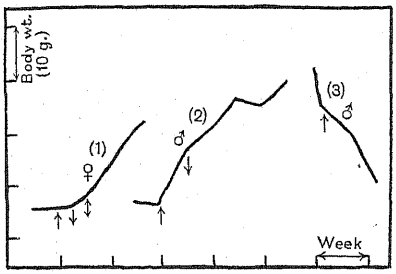


Fig. 3.

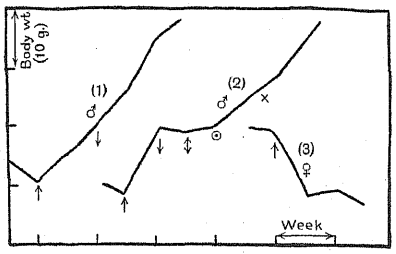


Fig. 4.

Fig. 3. Electrolysis at p_H 8·5 (Exp. 2).

Curve (1). Middle fraction; ↑ dose≡3 times original day-dose; ↓ dose≡4·5 times original day-dose; ↕ dose≡6 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡8 times original day-dose; ↓ dose≡4 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡12 times original day-dose.

Fig. 4. Electrolysis of a chemically purified fraction at p_H 8·5 (Exp. 3).

Curve (1). Middle fraction; ↑ dose≡2 times original day-dose; ↓ dose≡1·5 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡1·5 times original day-dose; ↓ dose≡1 original day-dose; ↕ dose≡1·25 times original day-dose; ⊙ dose≡1·5 times original day-dose; × dose≡2 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡4 times original day-dose.

Exp. 3. Electrolysis of a chemically concentrated preparation of vitamin B_1 at p_H 8·5.

This moderately concentrated preparation was obtained from brewer's yeast approximately by the method previously described [Guha, 1931]. The solution was active in a day-dose of 0·25 cc. and contained 1·5 mg. solids (1·3 mg. organic) per day-dose. 21 cc. were taken, adjusted to p_H 8·5, diluted to 50 cc. and electrolysed for 40 mins. with an average current of 0·2 amp. at about 10°. The solutions in the side chambers were treated as in Exp. 2. Fig. 4 shows that nearly 40 % of the vitamin migrated to the cathode under these conditions, which is in agreement with the earlier experiment.

Exp. 4. Electrodialysis of crude yeast extracts at p_H 7 and 4.

A crude yeast extract was made by extracting 1055 g. of yeast with boiling water. The filtrate, 1600 cc., was electrolysed for 12 hours at p_H 7 (1·5 amp.). About 30 % of the vitamin B_1 activity passed to the cathode. The cathode solution contained no vitamin B_2. The solution from the middle compartment was still able to maintain growth of rats totally deficient in the "vitamin B-complex" (Fig. 5). On further electrolysis at p_H 4 there was a loss of vitamin B_2 activity from the middle compartment, with no corresponding increase in activity in either of the side compartments.

Table I summarises the results obtained.

Table I.

Exp.	Conditions	Result	Total organic solids (g.) per day-dose: In the middle chamber before electrolysis	Total organic solids (g.) per day-dose: In the chamber containing the active fraction after electrolysis
1	Lead filtrate electrolysed at p_H 7	Cathode fraction inactive		—
		Anode fraction inactive	0·036	—
		Middle fraction active		0·037
	Further electrolysis at p_H 4·4	Cathode fraction active		0·018
		Middle fraction slightly active	0·037	—
		Anode fraction inactive		—
2	Re-electrolysis at p_H 8·5 of the active cathode fraction obtained in Exp. 1	Anode fraction inactive		—
		Cathode fraction, 50 % activity	0·018	0·024
		Middle fraction, 50 % activity		0·060
3	Chemically concentrated fraction electrolysed at p_H 8·5	Anode fraction inactive		—
		Cathode fraction, 50 % activity	0·0013	0·0012
		Middle fraction, 40 % activity		0·003
4	Electrolysis of crude yeast extract at p_H 7	Anode fraction inactive		—
		Cathode fraction, 30 % activity	—	0·014
		Middle fraction, 60–70 % activity		0·018

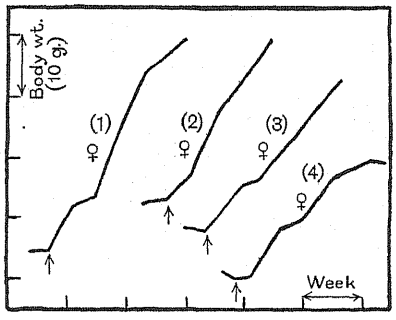


Fig. 5. Electrolysis of crude yeast extract at p_H 7.

Curve (1). Vitamin B_1-deficient rat; cathode fraction; ↑ dose≡3 g. fresh yeast.
Curve (2). Vitamin B_1-deficient rat; middle fraction; ↑ dose≡0·75 g. fresh yeast.
Curve (3). Vitamin B_2-deficient rat; middle fraction; ↑ dose≡3 g. fresh yeast.
Curve (4). Vitamin B complex-deficient rat; middle fraction; ↑ dose≡2 g. fresh yeast; growth falls off later.

Discussion.

From the results of Exps. 2 and 3 it is obvious that vitamin B_1 behaves like a base even at p_H 8·5, and, therefore, must be either a true base or an ampholyte whose isoelectric point is higher than p_H 8·5. Considering the latter case, such an ampholyte might be of the type carrying an excess basic group, or one whose basic dissociation constant is much greater than its acid dissociation constant.

Concerning Exp. 1 with lead filtrate, the fact that the vitamin did not

migrate at p_H 7 and did so at p_H 4·4 can probably be explained by taking into account the resistance of the cell at these p_H values. The much lower resistance at p_H 7 (noted by higher current passing) is no doubt due mainly to the fact that weak acids or ampholytes acting as acids at this p_H value exist as their ionised barium salts so that the barium cations may carry the major part of the current while at p_H 4 they are non-ionised and the barium is precipitated as barium sulphate. Hence the cations at p_H 4 are mostly bases other than barium, and so a much greater proportion of the current is carried by the vitamin. Owing to this variation in resistance of the cell at different p_H values due to the presence of large and varied amounts of ionised impurities, we did not succeed in getting even an approximate idea of the p_K value of the vitamin, since the experimental technique here adopted did not permit of a sufficiently adequate control of all the variables. From experiments with the crude yeast extract it is seen that the vitamin even in the crude state behaves like a base. The fact that the vitamin migrates in the crude state at p_H 7 appears to be in conflict with the result of Exp. 1 but the conditions of the two experiments are not comparable, as the total amount of current passed and the quantity and variety of ionised substances present are not the same. It was found possible to obtain vitamin B_1 in the cathode compartment practically free from vitamin B_2, but it was not possible to obtain vitamin B_2 free from vitamin B_1, as apparently there was a loss of activity of the former during the course of the electrolysis. This loss was probably due to the adsorption of vitamin B_2 on the precipitate of barium salts, proteins, *etc.*, which formed in the middle compartment. Very little of vitamin B_1 is apparently destroyed by electrolysis. Appreciable concentration may be obtained by this method at some of the earlier stages of fractionation.

Summary.

1. Vitamin B_1 in both the crude and relatively purified state behaves as a base even at p_H 8·5.

2. By electrolysis of a crude yeast extract, vitamin B_1 can be concentrated in appreciable quantities free from vitamin B_2 at the cathode.

Our thanks are due to Sir F. G. Hopkins for his interest in this work and to Dr L. J. Harris for valuable suggestions. We wish also to thank Mr A. Ward and Miss R. Leader for the care of the animals. One of us (T. W. B.) is indebted to the Medical Research Council for a grant.

REFERENCES.

Guha (1931). *Biochem. J.* **25**, 931.
—— and Drummond (1929). *Biochem. J.* **23**, 880.
Kinnersley and Peters (1928). *Biochem. J.* **22**, 419.
Williams and Waterman (1929). *Proc. Soc. Exp. Biol. Med.* **27**, 56.

CLII. BIOCHEMICAL LESIONS IN VITAMIN B DEFICIENCY.

By NICOLAI GAVRILESCU and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(*Received July 7th, 1931.*)

Several attempts have been made in the past to correlate the symptoms of vitamin B deficiency with failure in the oxidations in the body. In so far as these have been made upon the tissues of polyneuritic pigeons, it is probable though not certain that the work has been actually performed upon the so-called B_1 constituent of the complex. It will not be necessary here to mention more than the salient features of the present position of this knowledge. Those who work with polyneuritic pigeons must soon become aware of the bright red appearance of the blood often present in pigeons showing symptoms. Dutcher [1918] and Findlay [1921] found a fall in catalase and glyoxalase content respectively in certain tissues of the body of pigeons deficient in vitamin B_1—the enzymic power not being restored by the addition of yeast extracts *in vitro*. Hence, vitamin B_1 could not be a co-enzyme for the systems in question. A series of further observations, Abderhalden, with Schmidt [1920], and with Wertheimer [1921] upon the oxygen uptake of tissues, Hess [1921] and Hess and Messerle [1921] upon the reducing properties of tissues for dyestuffs [see also Vasarhelyi, 1926; Roelli, 1923] led to the conception that the fault lay in some failure in tissue oxidations. Some of this earlier evidence does not bear the light of modern criticism; other experimental work has not supported it, in especial the work of Terroine and Roche [1925], of Roche [1925], and of Marrian and Drummond [1926], who obtained no evidence of interference under their conditions either with the oxidations of the animal as a whole, or of the respiration or behaviour *in vitro* to dyestuffs of tissues taken from deficient animals. Though the earlier work is not convincing as it stands, it is not clear that later researches have been performed under precisely the same conditions as those of the original workers. As stated by Gugler [1928], the possible variations in technique are very large. Further it was pointed out by Kinnersley, Peters and Reader [1928] that the symptoms of opisthotonus in the pigeon might be associated with an oxidative deficiency in some small part of the brain, insufficient in weight to be detectable in the oxidation of the tissues as a whole. In this paper it is believed that evidence has been obtained in proof of this opinion. In recent work, Kinnersley and Peters [1930] have found increased lactic acid present, especially in the lower

parts of the brains of pigeons at or approaching the stage of symptoms. This suggested some enzyme deficiency in these parts.

In the following communication it is demonstrated that birds showing symptoms of head retraction, and birds in which these symptoms are threatening, show lowered powers of oxygen uptake *in vitro* in certain parts of the brain, which are marked in the lower parts of the brain. The lowering of oxidation is not general, because the cerebellum shows no such changes. In birds which have been dosed with vitamin, but in which no rise in weight has taken place, the tissue shows a power of oxidation approximating to the normal. This shows that the depression of oxidation found in the avitaminous bird is not merely associated with the state of nutrition as judged by the weight, but is some expression of the avitaminosis.

Experimental.

The oxygen uptake of brain tissue *in vitro* has been investigated by Loebel [1925], Meyerhof and Lohmann [1926], Warburg, Posener and Negelein [1924], Holmes [1930], who have found in rat and frog brain rather large and variable oxygen uptakes, lasting for several hours, which could be maintained at approximately initial intensity for some time by addition of glucose and lactate solutions to the Ringer's solution in which the tissue was suspended. The values obtained by these authors were greater than those recorded by Abderhalden and Schmidt [1920] and Roche [1925]. The latter however worked with the whole brain, mashed and suspended in moist air. It has been shown that white and grey matter have quite different rates of oxygen uptake, Holmes for instance gives 1200 mm.^{3} per g. per hour for brain grey matter, and 300 mm.^{3} per g. per hour for white in presence of air [see also Dickens and Šimer, 1930]. For the purposes of this research there are good reasons for obtaining these measurements with the pigeon, as so much is known about the behaviour of this animal in vitamin B deficiency. It is not however practicable to obtain measurements with the cortex and white matter of the pigeon's brain separately, in fact in the case of most parts of the brain the mixture of grey and white could not be properly separated. Upon this account, our procedure has been to guillotine the pigeons as previously described [Kinnersley and Peters, 1930], remove the two halves of the brain and separate the following parts, the cerebrum, cerebellum, optic lobes and remainder. The tissue has then been finely mashed by the use of small bone spatulas, before transferring to the vessels for analysis. The brain tissue of the pigeon can be rapidly divided in this way, and it is believed to be more satisfactory than other methods for this purpose. If the operations are carried through quickly, little loss of water occurs. After shaking at 38° such brain tissue is found to be finely divided. We have tried to slice as recommended for other tissues, but have not found it satisfactory in the case of the pigeon's brain.

The oxygen uptake[1] was determined in apparatus of the Barcroft type (as modified by Dixon and Elliott [1930]). The corked bottles containing Ringer's solution (3·0 cc.) were weighed before introduction of the tissue and re-weighed after placing the tissue in the fluid, from which the weight of tissue was calculated. The time from the guillotining of the bird to that of placing the apparatus in the thermostat (38° + 0·1) was 12–17 minutes, according to the number of observations in each experiment. The speed of operation was much enhanced by the use of the recent type of air-damped Sartorius balance.

The chemicals used in the research were of A.R. quality. The birds used were Homer pigeons and were fed according to the usual technique employed in this Department. The earlier experiments were done with a Ringer's solution of composition NaCl 0·9 %, KCl 0·025 %, $CaCl_2$ 0·030 %, $NaHCO_3$ 0·015 %. All others with a mixture of 80 parts Ringer with 20 parts $M/2$ acid potassium phosphate, to which was added sufficient NaOH to bring the p_H to 7·4. The precipitate which forms under these conditions was removed before use. It is realised that this mixture may not be ideal for the pigeon, and that research is needed as to the exact composition of the salts for this animal: since all the experiments were strictly comparable, it is not likely that error in the conclusions has been introduced in this manner.

Variations observed and discussion of the errors of the observations.

Despite the trouble which we have taken to work under constant conditions, we have been unable to reduce the occasional variations between duplicate samples of the same normal tissue to less than ± 5 %, though in the majority of cases duplicate observations have shown closer agreement than this. Though we have noticed that the figures of other workers have shown such occasional wide variations, we have tried to discover some of the sources of the variation. For samples of tissue from cerebellum, optic lobe and the rest of the brain, some of the variations are undoubtedly to be ascribed to the difficulty of sampling the mixed tissue: this is not so likely to apply to the cerebrum. Pieces of tissue larger than 0·12 g. tend to give low results, but there appears to be no marked difference in the behaviour of tissue which had been taken from the bird and sampled at room temperature or which had been allowed to cool in ice previously. It is very likely, though not proven here, that many of the variations are due to the failure to control the extent to which the dying cells liberate substrates other than the one studied for the enzyme systems present, or expose enzymes to the action of the substrate studied. As it was clear that the main object of our work was to reveal differences in the behaviour of normal and abnormal tissue, if such existed, we have made no attempt to do more than work under standard conditions, which would make our estimations comparable. In most of the experiments

[1] The constants of the apparatus were determined at approximately 12°, using a modification of Hoffman's method, and corrected for 38° by Warburg's formula. This method was found to be in substantial agreement with direct calibration of the apparatus at 38° by Hoffman's method.

for instance we have worked with oxygen at atmospheric pressure inside the bottles, though we are aware that the oxygen uptake can be increased under our conditions by the use of higher concentrations of oxygen. It is hoped to consider this question later. Meanwhile control experiments have shown us that our conclusions are not influenced by the pressure of oxygen employed. By working with air, we have been able to reduce substantially the time elapsing between death of the animal and the first observations, thereby minimising the possibility of other changes. For a similar reason, no attempt has been made to work with CO_2 present.

The tables show the values obtained in full, and the figures the mean of the recorded estimations, expressed in mm.^{3}/g./hour. The numbers can be readily reduced to Warburg's method of expression [1930] by taking the dry weight as approximately 20 % of the wet weight: *i.e.*

$$1000 \text{ mm.}^3\text{/g./hr.} = Q_{O_2}\ 5;\ 2000 = Q_{O_2}\ 10 \textit{ etc.}$$

Oxygen uptake of pigeon's brain in vitro *in mammalian Ringer's solution without additions.*

In some preliminary experiments performed by one of us in collaboration with Mr R. B. Fisher (April, 1930), the value for the oxygen uptake of normal pigeon's brain tissue in unbuffered mammalian Ringer's solution was found to be in general agreement with those of others. The oxygen uptake was followed for several hours after death and was found to be fairly constant for 30–90 mins., tending to show a slight diminution from 90–120 mins. The values for cerebrum (1000–1380) mm.^{3}/g./hr. were greater than those for the midbrain and optic lobes (1040), and these again greater than for the "rest" (730–930). This difference has been found to persist throughout the experiments. It is not certain that it is due only to the relative proportions of white and grey matter. Comparison of the normal and avitaminous birds revealed little difference between the two, except an indication of a fall for the optic lobes. As the object of our work was to determine the differences between the normal and abnormal, we restricted the subsequent periods of observation to 30–90 minutes after death.

Oxygen uptake of pigeon's brain tissue in vitro *in phosphate-Ringer with addition of glucose (0·25 %).*

In these experiments, the following technique was adopted uniformly. The apparatus containing the samples of tissue was placed in the bath 12–17 minutes after death. It was then shaken for 10 minutes with the taps open, after which the taps were closed. Readings were then made over a period of 60 minutes and the oxygen uptake for the sample considered to be the average uptake of oxygen during the period in question. In the majority of experiments the readings during the hour were consistent; there was a tendency in some experiments with the avitaminous "lower parts" for a slight fall towards

the end of the hour period. Though there was only a small difference between the normal and avitaminous birds for the oxygen uptake in the preliminary experiments, in which Ringer's solution alone was used, the difference is accentuated in the presence of glucose. Table I shows the actual values obtained in the various experiments. In Fig. 1 there is shown upon the chart

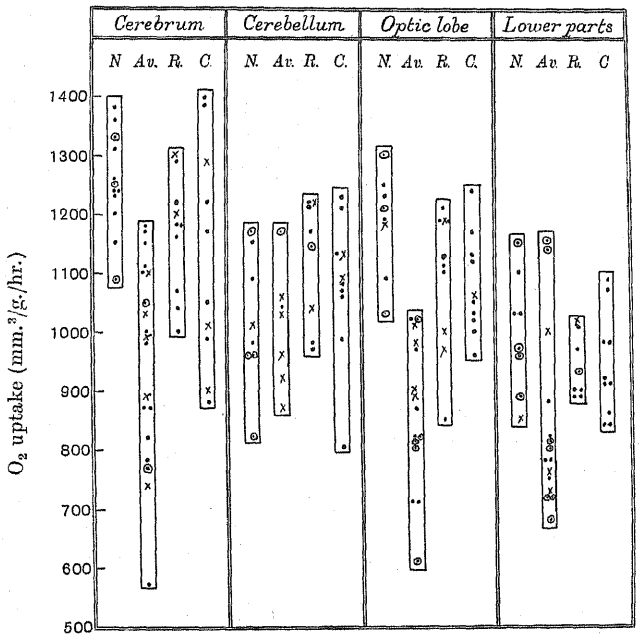


Fig. 1. Oxygen uptake *in vitro* of pigeon's brain (mm.^{3}/g./hr.). *N.* = normal; *Av.* = avitaminous; *R.* = rice fed; *C.* = cured avitaminous. • Average of two estimations agreeing within ±5 %. ⊙ Single determination. × Average of two estimations, not agreeing with ±5 %.

the average figures in the experiments. It will be seen that the values for optic lobe, cerebrum and the lower parts are reduced for the avitaminous birds; for cerebrum and optic lobes by some 20 % and for lower parts by 20 %, on the average. The values are about the same however for cerebellum. Though there appears to be no doubt about the conclusions for optic lobe and cerebrum, it was possible that the value for the lower parts might not have been significant. The matter has been kindly examined for us by Dr R. A. Fisher (Harpenden) by statistical methods. He has tested normal *v.* avitaminous for cerebrum and for lower parts and regards the difference for cerebrum as clearly significant: for lower parts he regards the difference as also significant, there being little probability of getting so large a deviation by chance. The figures prove that there is a difference in certain oxidation systems between normal and avitaminous brains, especially marked in cerebrum and optic lobes, and not present in the cerebellum. Examination of the chart will show that there was a marked tendency for bad duplicates in the samples from avitaminous brain. This must surely be significant and seems

Table I. *Values for oxygen uptake of*

N. = normal; *Av.* = avitaminous; *R.* = rice fed;

Cerebrum				Cerebellum			
N.	*Av.*	*R.*	*C.*	*N.*	*Av.*	*R.*	*C.*
1410 / 1360 (1)	1000 / 970 (4)	1260 / 1330 (6)	1100 / 930 (53)	1170 (3)	1170 (11)	1210 / 1240 (15)	1070 / 1120 (53)
1090 (9)	770 (8)	1200 / 1170 (15)	980 / 1000 (52)	820 (9)	1000 / 740 (14)	1230 / 1120 (70)	790 / 810 (57)
1330 (10)	1050 (11)	1030 / 970 (70)	1030 / 880 (63)	960 (12)	970 / 1150 (52)	1140 (73)	1090 / 1060 (63)
1250 (12)	1110 / 950 (14)	1270 / 1140 (73)	910 / 860 (65)	960 (10)	1030 / 920 (55)	1340 / 1100 (83)	1050 / 1080 (65)
1360 / 1360 (13)	1170 / 1180 (27)	1420 / 1190 (83)	1240 / 1200 (69)	1090 / 1100 (13)	990 / 850 (56)	980 / 970 (72)	1210 / 1050 (69)
1250 / 1220 (16)	870 / 870 (52)	1120 / 1020 (72)	1010 / 1100 (71)	1200 / 1100 (16)	1120 / 950 (78)	990 / 1100 (54)	990 / 1000 (71)
1300 / 1320 (62)	790 / 1010 (52)	1160 / 1160 (54)	1460 / 1340 (75)	940 / 1090 (62)	1150 / 1020 (80)	990 / 970 (58)	1160 / 1100 (75)
1210 / 1320 (20)	1150 / 1050 (55)	990 / 1090 (58)	1190 / 1050 (76)	990 / 970 (86)		1210 / 1240 (15)	1180 / 980 (76)
1260 / 1220 (21)	860 / 890 (56)	1250 / 1190 (44)	1360 / 1420 (77)				1200 / 1220 (77)
1250 / 1240 (88)	520 / 500 (61)	1200 / 1170 (15)	1230 / 1360 (96)				1250 / 1220 (96)
1190 / 1120 (89)	640 / 850 (64)		1080 / 1140 (51)				1000 / 1080 (51)
1180 / 1220 (90)	1210 / 990 (66)						
	1140 / 1170 (78)						
	1050 / 940 (80)						
	1200 / 1160 (84)						
	840 / 810 (87)						
	780 / 790 (91)						
Average: 1260	946	1162	1113	1032	1004	1122	1082

to indicate, as do the results for the different parts, an uneven distribution of certain catalysts.

The differences observed between normal and avitaminous brains are not of importance so far as the phenomena of avitaminosis B_1 are concerned, unless it is found that such differences are not merely due to the lowered state of nutrition—we purposely avoid the use of the word inanition. An avitaminous bird under consideration has lost some 40 % of its normal weight, if

pigeon's brain in vitro (*mm.*^{3}/*g./hr.*).

C. = cured avitaminous. Figures in brackets are exp. no.

Optic lobe				Lower parts			
N.	*Av.*	*R.*	*C.*	*N.*	*Av.*	*R.*	*C.*
1300 1270 (1)	710 720 (4)	870 840 (6)	1070 970 (53)	890 (3)	680 (18)	930 (15)	940 895 (53)
1270 1230 (3)	860 890 (8)	1250 1130 (15)	910 1010 (57)	1150 (9)	810 (11)	860 920 (70)	860 820 (57)
1300 (9)	610 (11)	1030 910 (70)	950 1040 (63)	970 (10)	790 740 (14)	1090 950 (73)	970 870 (63)
1210 (10)	820 990 (14)	1090 1110 (73)	990 1130 (65)	960 (12)	880 880 (27)	870 930 (83)	860 820 (65)
1030 (12)	810 830 (27)	1230 1110 (83)	990 1080 (69)	1020 1050 (13)	800 770 (52)	900 900 (72)	1000 970 (69)
1050 1310 (13)	1140 920 (52)	1170 1250 (72)	1190 1060 (71)	1030 (16)	830 690 (55)	910 870 (58)	820 910 (71)
1200 1180 (66)	830 910 (55)	1100 900 (58)	1190 1120 (75)	780 920 (62)	780 690 (56)	940 1000 (54)	1060 910 (75)
1050 1130 (62)	710 710 (56)	1080 1160 (54)	1210 1274 (76)	1090 1110 (86)	720 (61)	990 1040 (44)	1110 1080 (76)
1050 1240 (86) 1140	800 (61)	980 1080 (44)	1470 1100 (77)		720 (64)		1080 1070 (77)
	820 (64)	1250 1130 (15)	1050 1060 (96)		800 (66)		930 890 (96)
	810 (66)		910 1060 (51)		1160 830 (78)		780 860 (51)
	930 1000 (78)				790 780 (80)		
	1030 1010 (80)				1140 (84)		
	1020 (84)						
Average:							
1185	864	1083	1083	997	814	940	929

we interpret its normal weight as the maximum weight reached by the bird feeding naturally upon a mixed corn diet. A method of control which has been used with success has been to feed a bird upon yeast extract together with the amount of polished rice consumed daily by a bird feeding naturally upon polished rice alone [Kon and Drummond, 1927]. Under these conditions the curves for weight loss of two such animals run closely parallel. In the circumstances of our experiments it appeared that the best control would be

a bird which had lost some 40 % of its weight, but in which deficiency of vitamin B_1 had been excluded[1]. It has been recently shown [Carter, Kinnersley and Peters, 1930], that birds at the minimum weight reached upon polished rice do not increase in weight when given a daily dose of our charcoal concentrates. We have therefore obtained the oxygen uptake for the different

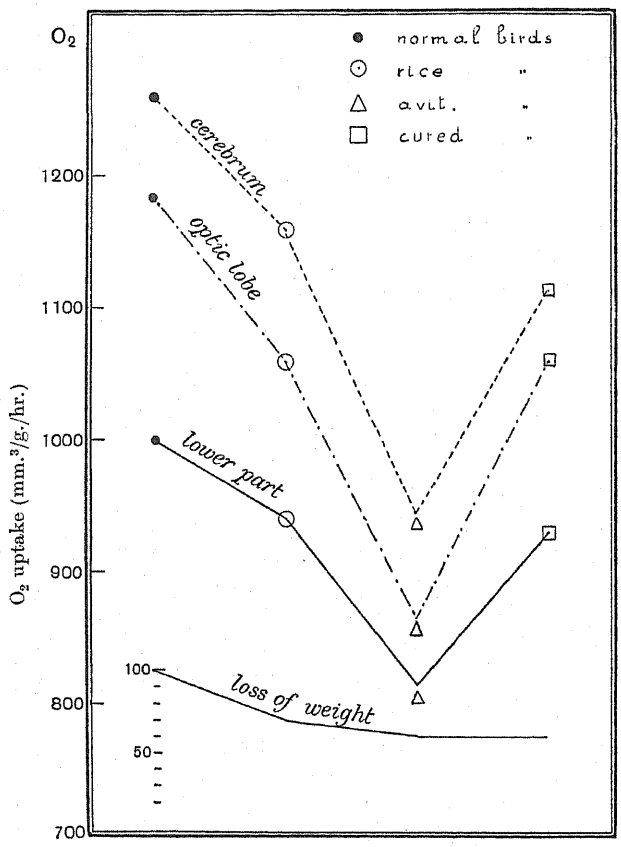


Fig. 2. Diagrammatic comparison of average oxygen uptake of different parts of the brain (pigeon) with the fall in weight at different states of avitaminosis. Ordinates, average oxygen uptake in mm.³/g./hr. Abscissae, periods in the feeding at which the average uptakes were observed. The interval between the first and second points represents 20–30 days; that between third and fourth points 24–72 hours approximately. The diagram includes a curve for the average weight at the same periods, showing that the average oxygen uptakes do not follow the fall in weight in the avitaminous periods.

parts of the brain in (*a*) birds which have been fed upon polished rice until the weight has dropped to some 40 % of the normal, to which doses of vitamin

[1] Throughout this paper we have made the assumption that the changes induced in the oxidations are produced by the vitamin B_1 in the extracts which cures the pigeon. This is justified in the present state of knowledge, because in some cases cures were induced with vitamin B_1 extracts of the *X* type (Appendix) [see Carter, Kinnersley and Peters, 1930]. It should always however be remembered that until a pure preparation of vitamin B_1 is obtainable, any conclusion of this nature can only be provisional.

have been in addition administered (rice in Table I), and (*b*) birds which have been fed upon rice until symptoms appeared and which have then been cured by addition of vitamin (cured in Table I). In some cases the vitamin has been administered for several days before carrying out the experiment.

The results of these experiments are also shown in Table I and Fig. 1, and the averages in Fig. 2. As would be expected the cerebellum shows no change. In the cerebrum, optic lobes and lower parts however dosing with the vitamin has given a rise of approximately 20 % in the rate of oxygen uptake. This proves conclusively that the lowered oxygen uptake is not a question of general inanition but is specifically associated with some entity in the vitamin extracts, which if not identical with vitamin B_1 must accompany it rather closely[1].

In grouping together the results for the avitaminous birds in head retraction and cured birds above, we have purposely overlooked certain minor differences which may influence the values. These are the length of time during which symptoms have persisted, and, in the case of the cured and rice-fed birds, the time elapsing between giving the dose and killing the bird. These points are partly discussed in the Appendix to this paper in which the protocols of the experiments are also given.

In drawing these conclusions as to the significance of the differences between the normal and avitaminous birds, we have considered the possibility that the water content of the brains might be different. This matter was extensively investigated by Roche [1925], who showed only very small differences, not amounting to more than about 2 %. In control experiments of our own we have obtained similar results.

The significance of the changes.

We feel that these results, together with the observations upon lactic acid in the brain [Kinnersley and Peters, 1930], show finally that the symptoms are central in origin in the polyneuritic pigeon. We have evidence of chemical change in the central nervous system on the one hand, and on the other find that the opisthotonus is relieved most quickly by injection of vitamin B_1 concentrates into the pigeon's cranium. From a theoretical point of view the changes might be due either to some general interference with the circulation in the brain, or to actual alterations in the tissue. The fact that the cerebellum does not appear to share in the general fall in oxidations (or in the changes in lactic acid) suggests that we are not dealing with a general failure of circulation. Though the condition of opisthotonus once established is progressive, it is wrong to consider such animals as moribund in the sense that no conclusions drawn at this time can be considered as valid indicators of vitamin B_1

[1] Reference to Fig. 2 shows that the oxygen uptake for the "cured" and "rice-fed" birds dosed with vitamin B_1 concentrates does not rise to the normal value completely. Though this may represent some change, we do not consider that the difference is sufficiently established by our experiments to warrant any far-reaching conclusions.

deficiency. Such birds show such a good blood pressure in many cases that the blood in the severed carotid arteries will spurt for some distance after the use of the guillotine. This leads to the conclusion that the differences in oxidations are not due to the lowered body weight or to failure of circulation. The localised nature of the trouble as well as of the increased lactic acid suggests that the differences are due to lack of some entity in the part affected. Kinnersley and Peters drew the conclusion that there was some change in the tissues of the brain, concomitant with the appearance of the symptoms, which led to accumulation of lactic acid first in the parts of the brain other than cerebrum and cerebellum and gradually involved the cerebrum. This picture seems to be in general true for the oxygen uptake (see Appendix). The other possibility that the depression of oxidation is due to some circulating toxic substance seems unlikely, both because such effects might be expected to be more evenly distributed, and also because, under the conditions of our experiments, the concentration of any such toxic stuffs in the tissues should be rapidly lowered by diffusion into the large volume of Ringer's solution. It seems therefore that the cause of the tissue changes can be one of three possibilities, a lowered content of either (*a*) certain enzymes (oxidases), or (*b*) certain essential substrates other than glucose, or (*c*) certain co-enzymes. The presence of the missing entity is dependent upon the presence of vitamin, as it is restored to the tissue by the cure of the bird. It is hoped that further work will decide whether it is the vitamin itself, or whether the vitamin is an intermediary in the production of some other substance.

Note. In a recent communication Vøgt-Møller [1931] has raised the question whether the symptoms of vitamin B_1 deficiency are due to the accumulation of methylglyoxal in the polyneuritic animal. The evidence for this is based upon the fact that liver extracts from avitaminous animals convert hexosephosphate to methylglyoxal, and not to lactic acid. While this is an interesting observation, in conformity with Findlay [1921], it would be dangerous to believe that lactic acid cannot accumulate in the avitaminous bird, until evidence to the contrary is produced. There is evidence now in the literature for the presence of increased lactic acid in the blood in birds [Collazo and Morelli, 1925; Kinnersley and Peters, 1930] and in man [Hayasaka, 1929], in the brain of the pigeon [Kinnersley and Peters, 1930] and in the tissues of the pigeon [Fisher, 1931]. In view of the possibility that methylglyoxal initially present in a trichloroacetic acid extract might be converted to lactic acid during the process of estimation, we have examined fresh trichloroacetic acid extracts of brain, blood and other tissues of the polyneuritic pigeon for the presence of methylglyoxal by the use of dinitrophenylhydrazine, but have not detected its presence. It can therefore be stated that any amounts of this substance, if present, can only be small; the upper limit would be set by the precipitation reaction with the reagent at about 1 mg. per 100 g. tissue. Since in the work of Kermack, Lambie and Slater [1927] it was found to be necessary to inject amounts of 0·7 g. of methylglyoxal per kg. into animals in order to produce symptoms, it seems unlikely that such small amounts even if present could be responsible for symptoms. As we have evidence of the presence of increased lactic acid, it therefore seems to be unnecessary at present to postulate that the effects are due to some other substance, the presence of which has not yet been demonstrated in the affected part.

SUMMARY.

1. Brain tissue from polyneuritic pigeons (vitamin B_1 deficiency) shows *in vitro* a lowered power of oxygen uptake in the presence of glucose as substrate. This is the case when symptoms are prolonged in all parts of the brain except the cerebellum.

2. Control birds of equal weight to the avitaminous, but previously dosed with vitamin B_1, and birds which have been cured by dosing with vitamin B_1 do not show the same depression of oxidation, which is therefore associated with the specific deficiency.

3. The symptoms of opisthotonus are associated with chemical changes in certain parts of the brain and are central rather than peripheral in origin.

We are indebted to the Government Grant Committee of the Royal Society, and to the Medical Research Council for grants towards expenses, to Dr M. Dixon for valuable help and to Dr R. A. Fisher for aid with the statistical examination of the figures. We are also grateful to Mr R. B. Fisher for the lactic acid estimations.

REFERENCES.

Abderhalden and Schmidt (1920). *Pflüger's Arch.* **185**, 141.
—— and Wertheimer (1921). *Pflüger's Arch.* **192**, 174.
Carter, Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832.
Collazo and Morelli (1925). *J. Physiol. Path. Gen.* **24**, 77.
Dickens and Šimer (1930). *Biochem. J.* **24**, 905.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Dutcher (1918). *J. Biol. Chem.* **36**, 63.
Findlay (1921). *Biochem. J.* **15**, 104.
Fisher (1931). *Biochem. J.* **25**, 1410.
Gugler (1928). *Biochem. Z.* **200**, 340.
Hayasaka (1929). *Tohoku J. Exp. Med.* **14**, 85.
Hess (1921). *Z. physiol. Chem.* **117**, 284.
—— and Messerle (1921). *Z. physiol. Chem.* **119**, 176.
Holmes (1930). *Biochem. J.* **24**, 914.
Kermack, Lambie and Slater (1927). *Biochem. J.* **21**, 40.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
—— —— and Leader (1928). *Biochem. J.* **22**, 276.
Kon and Drummond (1927). *Biochem. J.* **21**, 632.
Loebel (1925). *Biochem. Z.* **161**, 219.
Marrian and Drummond (1926). *Biochem. J.* **20**, 1229.
Meyerhof and Lohmann (1926). *Biochem. Z.* **171**, 425.
Peters (1930). *J. State Med.* **37**, 38.
Roche (1925). *Arch. int. Physiol.* **24**, 413.
Roelli (1923). *Z. physiol. Chem.* **129**, 284.
Terroine and Roche (1925). *Arch. int. Physiol.* **24**, 356.
Vasarhelyi (1926). *Pflüger's Arch.* **212**, 284.
Vøgt-Møller (1931). *Biochem. J.* **25**, 418.
Warburg (1930). Metabolism of tumours. (Constable.)
—— Posener and Negelein (1924). *Biochem. Z.* **152**, 309.

APPENDIX.

In Tables II, III, IV are recorded certain of the essential points from the protocols of the experiments, especial attention being drawn to points of interest in connection with the symptoms. In the references to dosing with vitamin B_1, it will be noted that cures have been effected with both the so-called *X* and *Y* preparations [Carter, Kinnersley and Peters, 1930]. The *X* preparations are relatively more free from vitamin B_5 than the *Y* preparations.

Table II. *Cured avitaminous birds (C. in Table I).*

Exp. No.	Bird No.	Sex	Days on diet	Loss of weight* %	Date of head retraction 1	Date killed 2	Weight on date 1 g.	Weight on date 2 g.	Lactic acid in blood at death mg./100 cc.	Remarks
51	753	—	22	36	Feb. 10	Feb. 10	172	172	—	Killed 5 hours after vitamin injection. 8 pigeon doses (*Y* preparation). 11.15 a.m., 12.15 p.m., no better. 2 p.m., symptoms cured. 4.15 p.m., practically well except that (1) vision was not completely restored, (2) not able to fly properly
57	456	—	45	47	„ 16	„ 17	280	264	71	7·5 pigeon dose (*X* preparation) injected on Feb. 16. Very fit when killed on Feb. 17
63	701	—	23	37	„ 18	„ 20	240	245	24	Feb. 18 and 19. 3 doses by mouth (*Y* preparation). When killed on 20th still showed weakness in wings, though head retraction completely disappeared on Feb. 19
65	412	♂	35	41	„ 17	„ 23	246	243	45	Feb. 17. 5 doses (*Y* preparation); Feb. 18, 19, 20, 21, 22, 3 doses daily by mouth. Altogether the bird received 24 day doses; bird rapidly recovered from symptoms and seemed well, but even when killed on 23rd, it still showed slight incoordination and difficulty in alighting properly
69	895	♀	22	29	„ 25	„ 26	167	173	34	5 doses (*Y* preparation) given 24 hours previous to death
71	635	♂	39	43	„ 25	„ 27	223	224	26	Feb. 25. 5 doses *Y*, Feb. 26, 3 doses
75	774	♂	37	51	Mar. 2	Mar. 3	258	233	—	Mar. 2. 15 doses (*X* preparation). 3rd flies fairly well. Kept in a dark box for 20 mins. before killing
76	476	♂	28	30	„ 3	„ 4	294	293	—	Mar. 3. 10 doses *X* preparation
77	870	—	32	49	„ 3	„ 4	205	210	—	Mar. 3. 10 doses *X* preparation
96	901	—	28	39	„ 26	„ 27	217	210	—	Mar. 26. 6 doses *X* preparation

* Loss of weight at time of symptoms. In some cases the lactic acid in the blood is not completely normal. This is probably connected with the amount of exercise, though from the results of Fisher [1931] it is likely that the high lactic acid represents a lingering vitamin B deficiency in other parts of the body. The doses were given by mouth, unless otherwise stated.

Note to Table II.

Of the above, the following gave oxygen uptakes below the average for the group of cured avitaminous birds. It is interesting to note that in practically all cases, some abnormality was still noticeable in the behaviour of the bird.

No.	Time after dose	Oxygen uptake: Cerebrum	Optic lobe	Lower part	Remarks
51	5 hours	Average	Low	Low	Vision not restored
57	24 hours	Average	Average	Low	Very fit
63	2 days	Low	Low	Average	Wings still weak
65	6 days	Low	High	Low	Some incoordination still present
71	2 days	Average	High	Low	No observation recorded

Table III. *Avitaminous birds with symptoms (Av. in Table I).*

Exp. No.	Bird No.	Days on diet	Weight loss %	Hours symptoms	Oxygen uptake*			Remarks
					Cerebrum	Optic lobe	Lower parts	
4	339	19	34	6	Average	Low	—	—
14	367	26	41	2	High	Average	Low	—
27	333	18	—	6	High	Average	Average	—
55	644	28	38	—	High	Average	Low	Emprosthotonus and weak
56	632	28	36	30	Low	Low	Low	Emprosthotonus for 30 mins. only. Symptoms threatening for 3 hours previously
74	920	19	34	—	High (1210)	High (1120)	High (1150)	Symptoms threatening only. O_2 uptake, mm.^{3}/g./hr.
84	415	21	30	2	High	High	High	Symptoms threatening only
87	980	24	34	30	Low	—	—	—

* In relation to average for avitaminous birds.

Notes. In this table are included those birds from Table I (avitaminous column) of which the time of onset of symptoms was known. The others were mostly birds which had developed symptoms overnight. In addition, experiment 74 has been added. With exception of 74 and 84, it will be seen that all birds showed a lower oxygen uptake in some part of the brain. In Exps. 14, 27, 55, a lowered oxygen uptake in optic lobe and lower parts was associated with a comparatively normal cerebrum value. This suggests that we are dealing with the same distribution as the lactic acid (Kinnersley and Peters). The lesion seems to commence in the part of brain other than cerebrum-cerebellum, and to extend to the cerebrum as symptoms are continued.

Exp. 74 shows that a bird with threatening symptoms may still show high oxygen uptake. Exp. 84 constitutes an exception in that it showed high oxidation together with symptoms. The interpretation is probably that the bird normally has a higher oxygen uptake than the usual normal.

Table IV. *Rice-fed birds (R. in Table I).*

Mostly dosed in addition with vitamin some hours or days before use.

Exp. No.	Bird No.	Sex	Days on diet	Weight loss %	Date of head retraction 1	Date killed 2	Weight on date 1	Weight on date 2	Lactic acid blood	Remarks
15	447*	—	23	—	—	—	—	—	—	Rice diet (not dosed with vitamin B_1)
44	382	—	30	39	Feb. 4	Feb. 4	290	290	—	Vision good at time of death
53	442	♂	23	32	,, 11	,, 11	265	265	26	10 doses B_1 (*X* preparation) given 90 mins. previous to death
54	732	♂	24	32	,, 11	,, 12	276	275	25	10 doses B_1 (*Y* preparation) on Feb. 11
58	655	♂	29	35	,, 16	,, 17	234	230	36	8 doses B_1 (*Y* preparation) on Feb. 16, 24 hours before death
70	628	♀	38	44	,, 23	,, 27	—	—	—	4 doses B_1 daily Feb. 23–27
72	722	♂	40	44	,, 22	,, 28	272	284	—	4 doses B_1 daily Feb. 24–27
73	814	♀	33	35	,, 22	Mar. 2	214	204	—	Feb. 22, 0·5 g. marmite+4 doses B_1 (*Y* preparation); Feb. 24–28, 4 doses B_1 daily
83	781	♀	41	40	,, 28	,, 4	283	260	—	Feb. 25, Mar. 3 and 4, 1 g. marmite

* Other data unfortunately missing.

CLIII. CARBOHYDRATE METABOLISM IN BIRDS.

III. THE EFFECTS OF REST AND EXERCISE UPON THE LACTIC ACID CONTENT OF THE ORGANS OF NORMAL AND RICE-FED PIGEONS.

By REGINALD BRETTAUER FISHER[1].

From the Department of Biochemistry, Oxford.

(*Received June 30th, 1930.*)

Kinnersley and Peters [1929, 1930] have demonstrated that there is an increase in the lactic acid content of the brain of the pigeon in polyneuritis (induced by feeding on polished rice) and that the increase is particularly marked in the mid- and hind-brain. They were inclined to interpret their results as an indication that a local defect in the metabolism of this lower part of the brain was responsible for the symptoms of polyneuritis, but it seemed to be of interest to investigate the lactic acid contents of other organs of the body in order to determine whether the phenomenon they observed was in fact a part of a more general disturbance. With this end in view a technique was developed for the use of iodoacetic acid as a "fixative" for lactic acid in the organs of the pigeon, in the hope that it might prove possible to obtain satisfactory estimates of the lactic acid contents of the pectoral muscle, the heart and the liver of the same bird. A preliminary account of this technique has already been published [Fisher, 1931].

In the course of an attempt to obtain standard conditions in the birds used for estimations it was discovered accidentally that the lactic acid contents of organs of polyneuritic pigeons depended to a considerable extent on the conditions of the experiment. This finding has been expanded, and in consequence the results, as presented, are not strictly complementary to those of Kinnersley and Peters. But they serve the original purpose in that they demonstrate that there is a definite general defect in lactic acid metabolism in the polyneuritic pigeon, in addition to the local defect described by Kinnersley and Peters.

Methods.

Lactic acid fixation. The use of iodoacetic acid as a fixative is based on Lundsgaard's [1930] original observation that lactic acid is apparently not formed in the stimulated gastrocnemius of a frog previously poisoned with the drug. The applicability of his assumption to normal pigeon muscle was

[1] Christopher Welch Scholar.

tested by the injection, intraperitoneally and intravenously, into a small series of pigeons of small amounts of sodium iodoacetate. Pectoral muscle from these pigeons was minced and incubated in $M/15$ phosphate buffer, p_H 7, at 37° for 2 hours. In three instances out of four the muscle showed a decrease in lactic acid content, and in the fourth a slight increase was observed. The decrease was not significant unless lactic acid were added to the muscle before incubation.

In view of the possible quantitative importance of the disappearance of lactic acid observed, experiments were made upon the effect of the addition of iodoacetic acid in buffered solution to finely minced unpoisoned muscle. This muscle contained initially from 400 to 550 mg. of lactic acid per 100 g. The rates of glycolysis observed in the presence of these high concentrations of lactic acid ranged from 120 to 610 mg. of lactic acid formed per 100 g. of tissue per hour, at 37°, when the muscle was suspended in buffer in a concentration of 1 g. of muscle to 5 cc. of buffer. But the range of rates of disappearance observed in the same system on the addition of iodoacetic acid was only from 40 to 70 mg./100 g. tissue. The results of these experiments are presented in Fig. 1. It should be noted that control experiments have

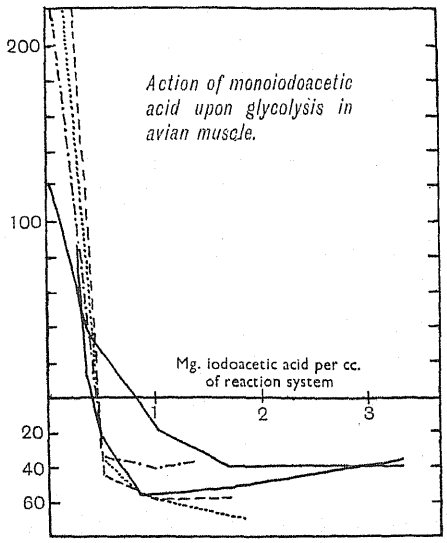


Fig. 1.

shown that no destruction of lactic acid occurs when lactic acid is incubated with iodoacetic acid in the absence of muscle tissue.

Fig. 1 indicates that, in a method of lactic acid estimation by fixation, the error which is produced by lactic acid destruction is likely to be very much less than the corresponding error introduced by glycolysis into the only other method available, *i.e.* rapid excision of tissues from a suitably anaesthetised animal, and immersion of the excised tissues in liquid air. But the figure

understates the case. Two rough experiments in which the iodoacetic acid content of a series of flasks was held constant, whilst the lactic acid content was varied by the addition of *dl*-lactic acid, showed that even at a concentration of 2·5 % total lactic acid (or 1·25 % *d*-lactic acid) the system causing the lactic acid disappearance was not saturated with respect to lactic acid. The rate of disappearance at this concentration was 100 % greater than that at a concentration of 0·5 % *d*-lactic acid. Since the concentration of lactic acid in the tissues taken for lactic acid estimation is in all probability about 0·01–0·02 %, judging by the figures obtained by Davenport and Davenport [1927] for guinea-pig and rat muscle, the rate of disappearance of lactic acid from the tissues under the conditions of *in vivo* fixation by iodoacetic acid should be too small to be of any account.

Since the data of Fig. 1 provide no guide to the best dosage for *in vivo* fixation of lactic acid by iodoacetic acid, the dose had to be found by trial and error. The only criteria available were the uniformity of the results and certain figures which had been obtained in a few preliminary experiments in which I had assisted Professor Peters. In these experiments the pigeons had been anaesthetised with "Liquid Dial," a proprietary non-volatile anaesthetic containing diallylbarbituric acid and urethane, and tissue samples had been removed as rapidly as possible and plunged into liquid air. The range of concentrations of lactic acid found is indicated in Table I. It is of interest to note the extremely high values for the hearts both of the normal and polyneuritic birds. The higher values were the more prevalent in both series, yet all the hearts were beating when they were excised, and it is doubtful if more than 3 secs. ever elapsed between excision and complete freezing. If these figures be compared with those in Table II it will be seen how extraordinarily rapid *post mortem* glycolysis must be in this tissue.

Table I. *Lactic acid content of pigeon tissues; liquid air method.*

Normal	Muscle: 22–35 mg./100 g. tissue
	Heart: 35–94
	Liver: 42–78
Polyneuritic	Muscle: 77–89
	Heart: 63–141
	Liver: 22–96

In all these experiments glycolysis must have occurred to some extent, so that any figure obtained by a successful iodoacetic acid method ought to fall below the lowest figure in the corresponding group in Table I.

Using this criterion it has been found that:

(1) "Dial" anaesthesia militates against success.

(2) Intravenous injection is unsatisfactory.

(3) Fixation is very unreliable in birds which die more than 12 minutes after the injection.

(4) Doses larger than 150 mg./100 g. body weight tend to cause stoppage of the heart before the drug has been adequately distributed.

The method finally adopted was to inject intraperitoneally a dose of 50 or 75 mg. of the drug per 100 g. body weight, the bird being unanaesthetised. The iodoacetic acid was dissolved in 5 % NaOH and made just alkaline to phenol red.

Of the total of 38 sets of estimations reported in this paper 19 were made using a dose of 50 mg./100 g., 18 were made using 75 mg., and one was made using a dose of 100 mg./100 g. of an impure commercial preparation. The higher dosage was used when it became apparent that the figures for the pectoral muscle obtained at the lower dosage were not satisfactory. However, as may be seen from Table II, the increase in dosage had little if any effect on

Table II. *Lactic acid concentrations in pigeon tissues: iodoacetic acid technique.*

1	2	3	4	5	6	7	8	9
		Dose of iodoacetic acid mg./100 g. body-weight	Lactic acid: mg./100 g.			Time to death in mins.	Length of exercise in mins.	Length of rest in mins.
Group	Exp.		Muscle	Heart	Liver			
Rested normals	1	50	6	6	8	4	None	25
	2	50	—	8	14	7	,,	37
	3	50	(39)	6	—	6	,,	37
	4	50	(24)	13	6	12	,,	130
	5	50	(71)	11	21	7	,,	31
	6	50	—	11	19	7	,,	80
	7	75	14	10	8	5	,,	52
	8	75	(25)	—	13	10	,,	45
	9	50	(139)	13	19	7	,,	75
Normals from cage	1	50	11	12	14	—	None	None
	2	50	13	16	15	—	,,	,,
	3	50	(136)	17	10	—	,,	,,
	4	50	12	18	—	4½	,,	,,
	5	50	(28)	13	9	9	,,	,,
	6	50	12	10	7	10	,,	,,
Exercised normal	1	50	15	14	24	12	8	None
	2	75	21	19	18	9	17	,,
	3	75	19	20	32	10	6	,,
	4	75	19	28	54	7	10	,,
Long rest avit.	1	50	—	11	24	7	None	309
	2	50	16	4	19	6	,,	409
	3	75	32	17	20	13	,,	336
Short rest avit.	1	50	41	24	37	6	None	96
	2	50	—	27	—	7	,,	42
	3	50	59	21	20	11	,,	28
	4	75	37	33	35	7	,,	45
	5	75	(133)	27	15	6	,,	26
	6	75	58	26	—	6	,,	150
Exercised avit.	1	75	68	83	70	10	6	None
	2	100	87	76	80	5	5	,,
	3	75	66	51	53	9	11	,,
	4	75	49	64	62	9	10	,,
Cured avit.	1	75	15	18	18	6	—	49
	2	75	13	24	23	6	—	67
Exercised cured avit.	1	75	26	27	37	12	9	None
	2	75	31	20	55	7	16	,,
	3	75	54	33	51	8	11	,,
	4	75	22	26	48	10	12	,,

the nature of the results. The general conclusion which may be drawn from the figures is that the method is eminently satisfactory as far as the heart is concerned, less satisfactory for the liver (though it is conceivable that the range of variation found in the liver corresponds to a true variation of the same magnitude), and of doubtful value for the pectoral muscle. It is interesting to note that the results indicate quite definitely that the drug affects the glycolytic systems of all these tissues.

Lactic acid determination. Samples of the tissues required (one sample from each pectoralis major muscle, the heart, and the greater part of the liver) were removed after the bird had died, pressed between filter-papers to remove adherent blood, and placed in tared 50 cc. centrifuge tubes, each of which contained 10 cc. of 10 % trichloroacetic acid. The tubes were weighed and the tissues were ground up with a glass rod. The tubes were allowed to stand for 45–60 minutes, and the fluid was poured off. The process was repeated three times, using water as the extraction fluid on the last two occasions, and the filtered extracts were collected in 100 cc. flasks. 10 cc. of 10 % $CuSO_4$ solution and 10 cc. of 5 % $Ca(OH)_2$ suspension were added to each flask, and the volume was made up to 100 cc. with washings from the filter.

After standing for an hour the extracts were filtered, and the first 10 cc. of filtrate were rejected. Estimations of the lactic acid content of 10 cc. portions of filtrate were made by the Friedemann, Cotonio and Shaffer [1927] method. *N*/200 iodine, freshly made up, was used for the final titrations, and Na_2HPO_4 was used to liberate the bound bisulphite, as recommended by Lehnartz [1928]. Duplicate titrations agreed as a rule to within 0·02 cc. As the net titration figure ranged from 0·20 cc. to 1·40 cc. the final figures are probably within 10 % of the true values. The method recovers 100–103 % of a standard zinc lactate solution of the order of concentration of the lactic acid in the extracts.

Preliminary treatment of the pigeons.

Polyneuritic pigeons. These birds were obtained by feeding on a polished rice diet and were used in the head-retraction stage. The precautions recommended by Kinnersley, Peters and Reader [1928] to ensure that the head-retraction was truly polyneuritic in origin were observed in each instance.

Cured polyneuritic pigeons. These were birds belonging to the polyneuritic group which had received 1–3 g. of marmite in water by the mouth from 12 to 24 hours before the injection of iodoacetic acid. In all instances the birds were free from any sign of polyneuritis when they were taken, and none of them developed any such sign during exercise.

Rest. The pigeon was kept at rest by wrapping it in a cloth and placing it in a dosing-box.

During the resting period the pigeon was kept in the dark [see, in this connection, Benedict and Riddle, 1929] and at the end of the resting period it was usually possible to take the pigeon out of the box without any move-

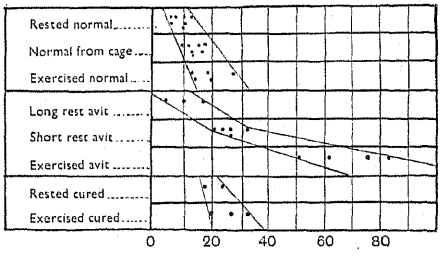
ment on its part, provided that a cloth were placed gently over its eyes before it was brought into the light, and it were grasped carefully with the cloth still obscuring its vision. The bird could be turned slowly on to its back and the injection made. It should be noted that the degree of rest is less in the polyneuritic birds than in the normal ones, since the head-retraction persists for some considerable time after they are placed in the dosing-box.

Exercise. It was found to be difficult to ensure that the extent of the exercise given to the birds should be comparable in all the classes (*i.e.* in normal, polyneuritic and cured birds). The normal and cured birds were exercised by being made to fly, either freely, by throwing them into the air, or else by holding them by the feet or tail and repeatedly drawing them downwards through the air. In these circumstances they beat their wings vigorously. The polyneuritic birds were exercised by allowing them free play for their convulsions and by tumbling them about on the ground. In this way normal and cured birds were probably exercised to the same degree, but the severity of the exercise of the polyneuritic birds was definitely less than that of the birds in the two other clases.

Results.

The lactic acid concentrations found in the pectoralis major muscle, the heart muscle and the liver tissue of eight groups of birds are presented in Table II. The figures in brackets in column 4 represent lactic acid concentrations which are in excess of those of the corresponding groups of Table I, *i.e.* results which are obviously not true values. Unfortunately it is impossible, on the data available, to apply the test afforded by Table I to all the groups.

In order that the significance of the variations in lactic acid concentration from group to group may be more readily appreciated, the figures for the lactic acid contents of the heart have been rearranged in Fig. 2 in a different



Mg. lactic acid per 100 g. tissue

Fig. 2.

form. It has already been said that more reliance is to be placed on these results than on those for the pectoral muscle and liver, but it will be found that what is true for the heart lactic acid content is true also, though not so immediately apparent, for the muscle and liver.

Fig. 2 demonstrates that the effect of severe exercise on the lactic acid content of the heart of the normal pigeon is surprisingly small. Since all the evidence goes to show that in severe exercise lactic acid is liberated in considerable amount, this seems to mean that in the 7–12 minutes which elapse between the end of exercise and the death of the pigeon practically all the liberated lactic acid is converted into some other substance. In the polyneuritic pigeon, on the other hand, we see that the lactic acid content of the heart remains very high for at least 5–10 minutes after exercise, is still twice as high as the normal content in birds from the cage which have been rested for 30–150 minutes, and falls to the level of the resting normal bird only after 300 to 400 minutes' rest.

The only simple interpretation of these results, having regard to their regularity, is that, whereas the normal pigeon possesses a remarkably efficient mechanism for the removal from its tissues of the lactic acid which accumulates in exercise, this mechanism is crippled in great part in birds suffering from avian polyneuritis. This crippling might be an adventitious effect, arising out of the partial starvation which is a consequence of the anorexia accompanying the disorder. Evidence that this is not so is afforded by the observations upon cured birds. There is very little difference between the behaviour of the lactic acid content of the heart in exercise in these birds and in normal birds, whereas there is an immediately apparent difference between its behaviour in cured birds and polyneuritic birds.

As a further proof that what has been termed by some authors "inanition" has nothing to do with the behaviour of the lactic acid content of the heart, the weights of the cured birds at the beginning of rice-feeding, at the time of dosing with the yeast preparation, and at the time of injection with iodoacetic acid, are given for all instances in which accurate data are available. It will be seen (Table III) that the lactic acid content has reverted to normal before any significant rise in weight has occurred.

Table III. *Effect of curative extracts on the weights of polyneuritic pigeons.*

	Weight of bird in g.		
No. of bird in Table II	Before rice-feeding	When dosed	When used
Exercised cured 2	342	230	230
Exercised cured 3	317	192	194
Exercised cured 4	330	251	260
Rested cured 1	365	239	246
Rested cured 2	332	221	228

It seems clear that none of the changes of weight after dosing can be considered to be significant, and that the differences observed between polyneuritic and cured birds must be ascribed to the yeast preparation (in all but one instance marmite) which cured the polyneuritis.

Discussion.

These results are of interest in connection with those obtained by Inawashiro and Hayasaka [1928] and Hayasaka [1929], using Japanese subjects suffering from beriberi. They showed that the blood-lactic acid concentration remained at a high level after severe exercise in beriberi patients for longer than in normal men and cured beriberi patients. They also showed that this tendency to persistence at a high level of the lactic acid of the blood developed progressively in a young male Japanese subject in whom avitaminosis B was induced by feeding him for 200 days on a deficient diet.

Their results conform exactly with the hypothesis suggested to explain the findings reported in this paper.

One point on which some doubt might be felt is the validity of the assumption that while in the normal bird a considerable amount of lactic acid is formed in exercise most of it disappears in the first few minutes of rest. No evidence has been cited in support of this assumption, but Kinnersley and Peters [1930] found 14–34 mg./100 cc. of lactic acid in the blood of pigeons killed with the guillotine (these birds corresponded to the "normals from cage" of this paper), and 80–126 mg./100 cc. in the blood of birds that had been severely exercised (the exercise was in most instances even more severe than that given to the pigeons used in this investigation). These figures are very valuable, since a number of investigations have established that in the absence of substances which inhibit cellular respiration glycolysis does not occur in avian blood. Rüter [1923] established this for goose blood, and Bornstein and Ascher [1926] found the same thing to be true for hen's blood. Gulland and Peters [1930] extended the observations to pigeon's blood. The figures given above may therefore be taken to represent the true lactic acid contents of the blood at the time of death, and it may be considered as established that there is a high concentration of lactic acid in the blood of a normal pigeon at the conclusion of severe exercise. Presumably therefore there must be a concentration of the same order in the organs of the pigeon from which the lactic acid passes into the blood. The fact that Kinnersley and Peters's figures for the blood-lactic acid are on the whole higher than the corresponding figures for the heart (comparing their "normals" with my "normals from cage" and their "exercised" birds with my "exercised avit." birds) can be accounted for if one considers, in the first place, that the exercise they gave their birds was probably more severe, and, in the second place, that the lactic acid of the solid organ is probably not distributed throughout the organ, but is present for the most part in the free water of the tissue. This free water comprises about 77 % of frog's muscle, but no determinations have been made on pigeon tissues [Hill, 1930].

The figures for exercised birds in Table II may therefore safely be considered to demonstrate that a rapid disappearance of accumulated lactic acid takes place in the first 5–12 minutes after the cessation of exercise in a normal

bird, whereas there can be only a very slow disappearance of lactic acid in the polyneuritic bird.

The difficulty encountered by Kinnersley and Peters in relating high lactic acid concentration in the brain to the symptoms of polyneuritis is somewhat diminished on this view. They found that they were able, by exercise, to raise the lactic acid content of the lower parts of the brain in normal birds to the level which they habitually found in the brains of polyneuritic birds. But the normal birds showed no sign of polyneuritis in these circumstances. However, it now seems that the lactic acid content can only momentarily be kept at that height in the normal bird, whereas in the polyneuritic bird it will tend to remain practically stationary for some time. It may be that the persistence of the lactic acid causes an accumulation of some precursor which is responsible for the symptoms. This would fit in with Vogt-Møller's suggestion [1931] that the symptoms of polyneuritis may be due to the accumulation of methylglyoxal, although even Sjollema and Seekles's [1926] comprehensive tabulation of the toxic effects of methylglyoxal does not permit any definite conclusion to be drawn. Peters and Gavrilescu (see note p. 1406) have been unable to isolate methylglyoxal from the brains of polyneuritic pigeons.

Summary.

A method is described for the fixation of the glycolytic systems in the organs of the pigeon by means of iodoacetic acid: the method is satisfactory for the heart, less satisfactory for the liver, and of doubtful value in most circumstances for the pectoral muscle.

Marked differences have been noted between the normal and the polyneuritic pigeon in the responses of the lactic acid contents of the heart, liver and muscle to exericse and rest. It is suggested that these differences signify that in the normal bird lactic acid formed in exercise is very rapidly removed from the tissues, but that in avian polyneuritis the mechanism of removal is very greatly crippled. This crippling appears to bear a close relation to the avitaminosis underlying avian polyneuritis.

REFERENCES.

Benedict and Riddle (1929). *J. Nutrition*, **1**, 528.
Bornstein and Ascher (1926). *Z. ges. exp. Med.* **52**, 607.
Davenport and Davenport (1927). *J. Biol. Chem.* **76**, 651.
Fisher (1931). *J. Physiol.* **71**; *Proc.* xxx.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Gulland and Peters (1930). *Biochem. J.* **24**, 91.
Hayasaka (1929). *Tohoku J. Exp. Med.* **14**, 85.
Hill (1930). *Proc. Roy. Soc. Lond.* B **106**, 477.
Inawashiro and Hayasaka (1928). *Tohoku J. Exp. Med.* **12**, 1.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
—— —— (1930). *Biochem. J.* **24**, 711.
—— —— and Reader (1928). *Biochem. J.* **22**, 276.
Lehnartz (1928). *Z. physiol. Chem.* **179**, 1.
Lundsgaard (1930). *Biochem. Z.* **217**, 162.
Rüter (1923). *Z. ges. exp. Med.* **37**, 151.
Sjollema and Seekles (1926). *Biochem. Z.* **176**, 431.
Vogt-Møller (1931). *Biochem. Z.* **233**, 248.

CLIV. THE BIOLOGICAL FILTRATION OF DILUTE SUCROSE SOLUTIONS.

By NEWTON WOOD BARRITT.

From the Department of General Microbiology, Rothamsted Experimental Station.

(*Received May 18th, 1931.*)

1. Introduction.

In a recent paper on this subject Jenkins [1931] has shown that in 0·1 % solution either pure sucrose, or mixtures of sucrose and lactic and acetic acids, are readily oxidised on a percolating filter containing $\frac{1}{8}$ to $\frac{1}{4}$ inch clinker to a depth of 54 inches when fed at a suitable rate of flow. The suitability of soluble carbohydrates for the nutrition of micro-organisms needs no emphasis and the more or less complete exhaustion of such solutions is only to be expected provided a sufficient number of the right type of organisms is present under suitable conditions for a sufficient period of time.

The fundamental factors in biological filtration are obviously

(1) the microbial population;

(2) the conditions under which this population exists as determined by

  (*a*) the size of the particles constituting the medium,

  (*b*) the rate of flow of the liquid;

(3) the concentration of the nutrient solution.

The object of these investigations was the more precise determination of the above factors and their application to the treatment of some 250,000 gals. per day of liquor containing 0·2 % of sugar in solution.

The rate of flow used by Jenkins was 9 cc. per minute, equivalent to 86 gals. per cubic yard of filter bed per day. The treatment of 500,000 gals. of a 0·1 % sugar solution at this rate of flow would require a filter bed five-sixths of an acre in area with a depth of 54 inches. It is obviously of some importance to know if these dimensions are the minimum required for the effective solution of the problem.

The complete oxidation of sugar to CO_2 and water requires 1·12 times its weight of oxygen, so that a 0·2 % sugar solution, *i.e.* 200 parts of sugar per 100,000, will require 224 parts of oxygen per 100,000 of water. Since the solubility of oxygen in water is 1 part by weight per 100,000, the solution will require constant aeration during its passage through the filter, and the rate at which this occurs will be a very important controlling factor in the efficiency of the filter. The rate of aeration is determined by the size of particle and the rate of flow of the liquid.

The semi-commercial scale experiments conducted at Colwick, and referred to by Jenkins, showed that the use of fine clinker of $\frac{1}{8}$ to $\frac{1}{4}$ inch was liable to lead to the choking of the filter bed and insufficient aeration, resulting in a very much reduced purification.

Investigations of the inter-relation between concentration of nutrients, the size of particles and rate of flow and their bearing on the rate of oxidation are described in the present paper together with an account of the nitrogen and phosphate requirements of the biological film.

2. The five-day test.

This test measures the polluting value of an effluent by means of the biological oxidation of the organic matter. It therefore achieves the object of the filtration process. For this reason a study of the test will help towards a better understanding of biological filtration.

A sample of the water to be tested is diluted with water saturated with air at atmospheric pressure and 18°. By choosing a suitable degree of dilution the water will supply sufficient oxygen for the oxidation of the organic matter, provided of course that the right organisms are present. Determinations are made by Winkler's method of the dissolved oxygen in the water at the beginning and after 5 days' incubation at 18°, the difference being the amount of oxygen used in the oxidation of the organic matter.

With the object of testing the degree of correlation between sugar content and oxygen absorbed, 5-day tests were carried out on 0·2, 0·15, 0·10 and 0·05 % sugar solutions. The results are given in Table I, dilutions of 1 in 200 being used in each case.

Table I.

Concentration of sucrose solution %	Dissolved oxygen available in parts per 100,000	Dissolved oxygen required in parts per 100,000 (Calculated)	Dissolved oxygen absorbed in parts per 100,000 (Actual)	Sucrose accounted for %
0·20	180	220	131	60
0·15	180	165	102	62
0·10	180	110	62	56
0·05	180	55	35	63

In every case the amount of oxygen absorbed is little more than half the amount required for complete oxidation of the sugar. This discrepancy may be accounted for by (*a*) sugar undecomposed, (*b*) sugar metabolised but not oxidised. To what extent each of these two factors is responsible for the low oxygen absorption would be very difficult to determine. Assuming the unabsorbed oxygen to be due entirely to undecomposed sugar the maximum amount of sugar would be 0·0004 %. Actually most of it will exist as decomposition products of sugar and as cell tissue of the organisms effecting the decomposition. A more extended incubation period is obviously necessary for more complete oxidation. At the extreme dilutions of sugar used in this test the generation time of the organisms must be very considerable, probably 12 hours

or more (see p. 1432). Another series of tests was carried out in duplicate with the same dilution using a 0·15 % sugar solution. Ten bottles were incubated in each case and the temperature of incubation raised from 18° to 25° during the first 5 days. Determinations of oxygen absorbed were made at intervals during 20 days. The results are given in Table II.

Table II.

	Present at start	Dissolved oxygen in parts per 100,000 after 1	2	3	4	5	6	7	10	20 days
I	142	92	55	35	27	24	24	22	19	2
II	142	94	56	35	26	24	24	23	19	2

These results show that the absorption of oxygen is most rapid during the first 3 days and that 70 % of the sugar is oxidised after 4 days' incubation. This result is considerably higher than that obtained in the previous experiment.

Cook and Stephenson [1928] using pure cultures of *B. coli* measured the oxygen absorption in a Barcroft apparatus and found that for glucose only two-thirds of the theoretical amount was absorbed by the organism. They offer no explanation to account for the incomplete oxidation of the glucose and do not consider that bacterial growth may be responsible. In the 5-day test, however, there is an increase in bacterial numbers during the first 2 days and the writer is of opinion that bacterial growth and synthesis is responsible for the incomplete oxygen uptake. The differential rates of oxygen uptake as well as the different amounts recorded by Cook and Stephenson [1928] for various carbohydrates seem to point to a difference in value of these carbohydrates in metabolic synthesis (see p. 1431). This subject is being investigated in greater detail.

The determination of the possible variation due to the inoculation was made by preparing a series of tests using tap water only for dilutions, and another series with the addition before dilution of 1 % of a laboratory filter effluent to the sugar solution to be tested. The results are given in Table III.

Table III.

No. of sample		Oxygen absorbed per 100,000	Standard deviation
1	Tap water only	91	Mean = 92±7·4
2		87	
3		89	
4		108	
5		87	
6		89	
7	Tap water *plus* 1 % filter effluent	112	Mean = 110±3·1
8		109	
9		115	
10		107	
11		113	
12		106	

These results show that leaving the dilution water to a chance inoculation causes a decreased oxygen absorption and a greater variation in the amount than using a definite inoculation from a filter bed.

The standard deviation of the mean using a definite inoculation is 3·1 which means that a single determination may vary as much as 9·0 % from the mean value.

As an accurate method of determining the amount of organic matter present in a liquid this test is obviously not very reliable. A controlled inoculation is not practicable since there is no known method of producing cultures of bacteria in a uniform state of efficiency.

The possibilities of a chemical test answering the purpose are not encouraging. Recent work at Rothamsted has shown the 4-hour permanganate test to be unsuitable for estimating the oxygen requirement of organic acids [Water Pollution R.B. Report, 1929]. The only reliable method is that of combustion, and this could not be considered as a true measure of the polluting effect, since there are organic substances which are comparatively inert biologically. There appears therefore to be no alternative to this incubation test, and, provided too much reliance is not placed on single determinations, it may be considered to answer the purpose for which it was designed. In the succeeding experiments it has been used as a measure of the amount of purification effected by biological oxidation on percolating filters. In this case the calculations are made from determinations obtained by using identical inoculations.

3. The biochemical oxidation of sucrose.

A very extensive literature exists on this subject which for the purposes of this paper may be summarised as follows.

After hydrolysis of the sucrose the hexose sugars give rise to methylglyoxal ($CH_3.CO.CHO$) which yields lactic acid ($CH_3.CHOH.COOH$) and then by oxidation pyruvic acid ($CH_3.CO.COOH$). Pyruvic acid on hydrolysis yields acetic and formic acids. Formic acid is readily decarboxylated yielding CO_2 and H_2. Alternatively pyruvic acid may be decarboxylated yielding acetaldehyde which on reduction yields alcohol and on oxidation acetic acid. Acetaldehyde may also form the starting-point of several organic syntheses by polymerisation and condensation forming higher fatty acids and alcohols.

In biological filtration any or all of these compounds may be produced according to the bacterial population and conditions of aeration of the filter bed. In any case the fundamental processes in all the different types of fermentation are the same, and the object of filtration, *i.e.* the complete oxidation of the carbohydrates to CO_2 and water, is best secured by reducing the synthetic reactions to a minimum. In accordance with Hopkins's dictum the production and accumulation of any particular compound in the filter bed, other than the final products of oxidation, is a measure of its uselessness to the organisms producing it [Stephenson, 1930]. From the point of view of

pollution the intermediate products, lactic and acetic acids, theoretically have the same oxygen absorption value as sugar, so that their production does not result in any reduction in the polluting effect of the liquid. Their decomposition requires the presence of hydrogen acceptors which may be provided either by free oxygen or oxygen combined as nitrate [Quastel, Stephenson and Whetham, 1925]. Since the solubility of oxygen in water is very slight, it may become the limiting factor determining the rate of oxidation. It has long been known that denitrification is part of the respiratory process of bacteria under conditions of restricted aeration. The presence of oxidised nitrogen either as nitrites or nitrates in a filter bed is generally considered as indicating its efficient working, but the possibility of their playing a part in the actual oxidation of carbohydrates does not appear to have been previously recognised.

4. Experiments with sectional filters.

With the object of obtaining more information as to the working of a biological filter, a special filter was designed by the writer and manufactured in earthenware by Messrs Doulton of Lambeth. The principle of this filter was adopted by Jenkins and described in a recent paper [1931]. The original apparatus consists of pipe sections each 1 foot in length and 4 inches in diameter fitted at one end with a perforated plate containing 5 holes $\frac{1}{2}$ inch in diameter. Each section was set on a separate earthenware saucer provided with a single drainage hole 1 inch in diameter. The object of the saucer was to collect the effluent from each section and deliver it to the centre of the next section below. In this way the possibility of the liquid passing down the inner surface of the pipes was obviated. A certain amount of spreading of the effluent over the surface of each section was effected by the presence of a ring of drip nipples round the lower edge of the drainage hole of each saucer.

The capacity of each section, assuming a 9 inch working depth, was such that when supplied with 1 litre of liquid per 24 hours it was equivalent to a filter bed receiving 100 gallons per cubic yard per day (g.y.d.). Five such sections placed in a column can be conveniently supplied with 5 litres of liquid per day from a 6-litre aspirator at this unit rate of feed. The possible criticism that such a filter would be subject to abnormal conditions on account of the supposed increased aeration due to the air gaps between each section is not valid. The contraction of the film in passing from one section to another would result in less aeration than if the filter medium were continuous. It should be remembered, however, that the choking of any one section not only affects that particular section, but in practice would also put out of action the whole column.

In filtration experiments the rate of flow of the liquid through the bed is obviously of great importance and precautions must be taken to ensure that the rate of flow is actually what was intended. In these experiments the aspirators were fitted with a Mariotte's constant head device which released

the liquid under a very low uniform hydrostatic pressure. The use of a "T" piece outlet obviated the possibility of gravitational pull by the dropping liquid and at the same time facilitated cleaning operations. The apparatus was kept free from both organic and mineral deposit by rinsing with hydrochloric acid each day. Before sampling, checks on the rate of flow were made by measuring the volume of effluent collected in 30 minutes.

A. *The size and nature of the filter medium.*

In this experiment two columns each of six sections were used. One column was filled with fine gravel of $\frac{1}{4}$ to $\frac{3}{8}$ inch and the other with clinker of $\frac{3}{8}$ to $\frac{5}{8}$ inch. Each column was inoculated with 100 cc. of a suspension of film obtained from a large scale experimental filter being operated at a Sugar Beet Factory. Both laboratory filters were fed with a solution of the following composition at the basic rate of 100 g.y.d.

			%
Sucrose	...	...	0·2
NaCl	...	...	0·03
K_2HPO_4	...	...	0·03
KH_2PO_4	...	...	0·02
$MgSO_4$	...	...	0·01
$(NH_4)_2SO_4$	...	...	0·03
$CaCO_3$	...	...	0·03 (supplied by the tap water).

The p_H was 6·8–7·0.

This experiment was run continuously for a period of 60 days. Samples of the effluents of each section were examined daily for p_H and mineral nitrogen and weekly for degree of purification by means of the 5-day test.

(*a*) p_H *values*. These were determined by means of the B.D.H. capillators, with bromocresol purple and bromothymol blue. The results were plotted as curves with the numbers of the sections as ordinates and the p_H values as abscissae. During the first week little or no change in the reaction of the liquid occurred in its passage down the filters so that the graph is almost a straight line. During the second week the p_H of the effluents from the second section became more acid (see Figs. 1 and 2).

By the end of the second week acidity developed in the effluent from the first section and continued to increase during the third and fourth weeks, but to a greater extent in the effluent from the gravel filter than in that from the clinker. This difference in acidity between the two filters continued throughout the remainder of the experiment. The p_H values of sections 1 and 2 of the gravel filter averaged 5·5 whilst in the corresponding clinker sections the average was 6·0. The effluents were always less acid from section 3 and generally reached neutrality in section 4. In sections 5 and 6 they became slightly more alkaline (p_H 7·0–7·1) than the original solution supplied from the aspirator. This development of alkalinity in the lower sections diminished after the 30th day with the increase in nitrification, more especially in the case of the

clinker filter in which the effluent gradually became more acid (p_H 6·5) after leaving section 4 (see Fig. 2).

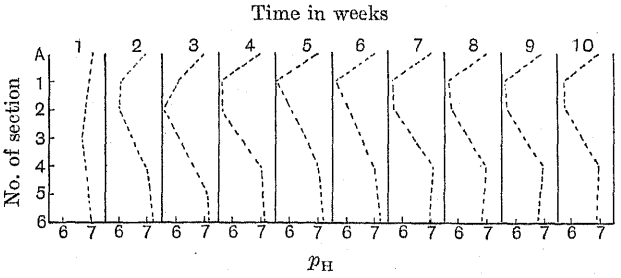


Fig. 1. p_H of gravel filter.

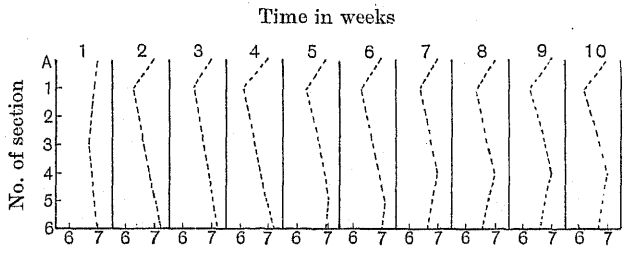


Fig. 2. p_H of clinker filter.

The greater acidity developed in the gravel filter may be due to deficient aeration, unless some basic material in the clinker was exerting a neutralising effect.

An attempt was made to diminish the acidity of the gravel filter by placing glass dishes filled with broken chalk beneath the drainage holes of sections 1 and 2. Although the chalk was at first rapidly corroded by the acid liquor there was no significant change in the reaction of the effluents. After two days the chalk was covered with a gelatinous film which prevented any further corrosion. On testing the liquid in the dishes containing the chalk it was found that the p_H was even less than that of the liquid dropping on to it from the section above. The same lowering of the p_H also occurred on collecting the effluent in glass dishes without any chalk. In 2 hours the p_H dropped from 5·5 to 5·0 and in 20 hours 4·2 was recorded. The liquid at the surface gave a p_H value of 5·8. This higher p_H of the surface layer could only be accounted for by greater aeration.

In order to confirm the improbability of any buffering effect of the clinker medium the basicity of the media was determined by titration with both hydrochloric acid and acetic acid.

With both mineral and organic acids the gravel contains more basic material than the clinker. The difference between the two is greater in the case of the organic acid on account of the occurrence of sulphides in the clinker which are not decomposed by acetic acid.

Time of shaking (hours)	Gravel	Clinker
	cc. of $N/10$ HCl acid neutralised	
24	7·3	5·8
48	9·0	8·8
72	9·4	9·15
	(Some H_2S was evolved by the clinker.)	
	cc. of $N/5$ acetic acid neutralised	
24	3·7	0·5
48	8·0	0·6
72	8·6	0·7

It must therefore be concluded that the difference in reaction between the two filters is due entirely to aeration and not to chemical composition. Jenkins [1931] states that "for acid to be produced the p_H of the nutrient solution must not be less than 6·7 to 6·8." It is difficult to reconcile this statement with the above observations or with any known theory of fermentation.

The cause of this difference in aeration must be attributed to the size of the particles of gravel. Although the total volume of interstitial space is independent of the size of particles, the permeability to water is proportional to the square of their diameter [Green and Ampt, 1912]. With small particles the interstitial space is also diminished to a greater extent by the film of liquid and bacterial slime. After running for 45 days the first section of the gravel filter was completely blocked with film and the liquid ran over the side of the section. This filter was therefore considered to be out of action since the lower portions only worked in virtue of their being sectional. The clinker filter continued working in a satisfactory manner for another month.

The gravel filter was therefore dismantled and the amount of film present in each section was determined in the following way.

(*b*) *Determination of weight of film in gravel filter.* The gravel from each section was shaken thoroughly with successive lots of 400 cc. of water and the total volume of suspended film made up to 2 litres. This was allowed to settle and 1 litre of clear water was decanted. The remaining litre of film suspension was then thoroughly agitated and 60 cc. of it were evaporated to dryness, dried at 100° and weighed again. It was then ignited and the ash determined.

The results are as follows.

Table IV.

Section	Total weight of film g.	Weight of ash g.	Weight of volatile matter g.	Ash %
1	14·6	4·8	9·8	33
2	14·0	6·2	7·8	44
3	11·4	6·2	5·2	54
4	9·4	5·2	4·2	55
5	12·6	7·2	5·4	57
6	5·2	3·4	1·8	65

These results show

(1) that the greatest amount of film occurs in the first section and that

the amount generally diminishes with depth; the irregularity of the fifth section is probably due to collection of film discharged from higher sections;

(2) that the ash generally increases in percentage amount with depth and constitutes the bulk of the film in the lower sections.

(*c*) *Reconstructed gravel filter.* This filter was then reconstructed with coarse gravel as follows:

Section 1, 2	$\frac{1}{2}$ to $\frac{3}{4}$ inch	Coarse.
,, 3	$\frac{3}{8}$ to $\frac{1}{2}$ inch	Intermediate.
,, 4, 5, 6	$\frac{1}{4}$ to $\frac{3}{8}$ inch	Fine as before.

It was then inoculated with the wash water from the previous medium.

As in the previous experiment acidity first developed in section 3 at the end of the first week, after which it occurred only in the first (p_H 5·6) and second sections (p_H 6·2). After the second week the p_H of the first section varied between 6·0 and 6·5 whilst that of the second section rarely fell below 6·5. The p_H curves obtained with this graded gravel were almost identical with those obtained with the clinker filter, thus confirming the conclusion that for the above experiment the size of the particles and not their chemical composition or texture determines the development and efficiency of the film.

Both filters continued to operate in a very satisfactory manner for 20 weeks when both were choked in the lower sections by copious discharge of film from the sections above.

(*d*) *Purification.* The purification effected by a filter is calculated from the oxygen absorption figures in the 5-day test of the original solution and of the effluent. The difference between these two determinations expressed as a percentage of the first is the percentage purification. The results obtained during the first period of the two filters are given in Table V.

Table V.

Section	Percentage purification 1st week	2nd	3rd*	4th	5th	6th	7th	8th
				Gravel filter.				
1	42	43	16	21	22	21	3·3	15
2	58	77	27	46	40	55	23	40
3	55	90	43	78	66	68	41	32
4	68	92	57	86	87	82	66	77
5	68	90	69	96	95	84	85	87
6	72	90	76	93	96·5	84·5	89	94
				Clinker filter.				
1	27	52	42	60	22	38	34	36
2	30	74	59	78	40	46	55	66
3	52	76	68	90	54	65	80	93
4	58	76	73	93	81	77	90	98
5	72	86	81	96	90	85	90	100
6	73	90	79	97	91	93	95	100

* Nitrogen omitted.

These results lead to the following conclusions.

(1) There was no difference between the filters during the first 3 weeks. Both gravel and clinker media gave 90 % purification after 2 weeks.

(2) Omission of nitrogen and consequent retardation of the growth of film lowered the efficiency immediately.

(3) Most of the purification was effected in the first three sections and very little effect was produced by the sixth section (see Fig. 4).

(4) The fine gravel filter showed a fall in efficiency in the first section after the second week, but without any appreciable effect on the lower sections.

The changes in the purification of each section are shown in Figs. 3 and 4.

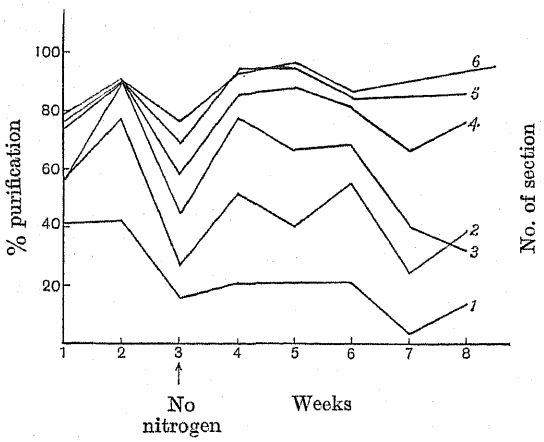


Fig. 3. Purification curves for fine gravel filter.

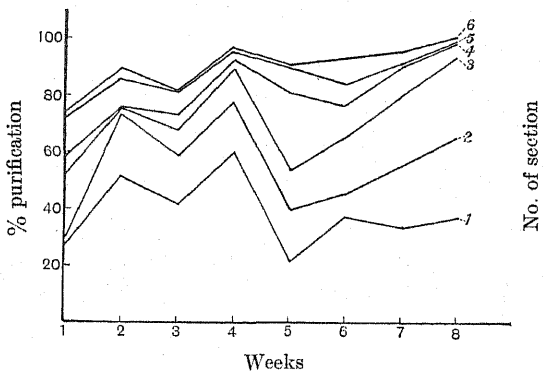


Fig. 4. Purification curves for clinker filter.

The purification results of the second period after reconstructing the gravel filter are as follows (Tables VI and VII).

These results show:

(1) that the coarse gravel ($\frac{1}{2}$ to $\frac{3}{4}$ inch) was much superior to fine gravel ($\frac{1}{4}$ to $\frac{3}{8}$ inch) and to clinker ($\frac{3}{8}$ to $\frac{5}{8}$ inch); the purifications in the first two

Table VI. *Coarse gravel (regraded).*

	Percentage purification							
Section	1st	2nd	4th	6th	9th	11th	13th	15th week
1	26	64	61	77	68	20*	46	69
2	45	88	74	86	85	73	69	90
3	60	90	84	91	86	85	82	92
4	75	94	93	95	89	95	92	95
5	83	94	95	95	96	95	95	95
6	85	95	94	95	96	95	97	97

* Drainage outlet closed by pebble.

Table VII. *Clinker.*

	Percentage purification					
Section	1st	3rd	5th	9th	12th	14th week
1	22	26	22	20	45	32
2	64	69	33	35	55	43*
3	80	85	59	40*	67	72
4	86	95	87	90	92	90
5	95	97	90	95	96	95
6	97	97	95	99	98	98

* Choked with larvae and debris, and cleared.

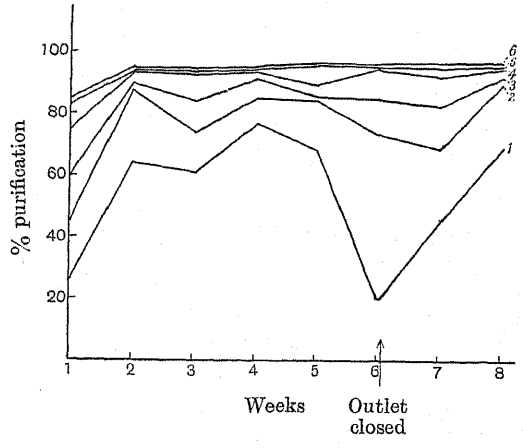


Fig. 5. Purification curves of regraded (coarse) gravel filter.

sections were much higher and showed less falling off in efficiency with age (Fig. 5);

(2) that starting the filter with the previous inoculation showed more rapid development of purification than with the original film from the large scale filter bed;

(3) that 3 feet of gravel ($\frac{1}{2}$ to $\frac{3}{4}$ inch) will give 90 % purification of 0·2 % sucrose solution after 2 weeks and maintain this for more than 16 weeks;

(4) that very little extra purification was obtained after the 4th section in either filter.

B. *Oxidation of lactic and acetic acids.*

The difference in acidity and purification between the coarse and fine filter media suggested a possible correlation between p_H and purification. An experiment was therefore carried out using 0·2 % solutions of lactic and acetic acids in place of sugar. The experiment ran for 14 days and was characterised by remarkably good purification figures and nitrification.

The results are given in Tables VIII and IX.

Table VIII. *Oxidation of lactic acid.*

			Purification %		
	p_H	$[H^·] \times 10^7$	2nd day	7th day	14th day
Aspirator	3·7	2000	—	—	—
Section 1	4·8	158	16·6	43	58
„ 2	6·5	3·2	63·0	83	83
„ 3	6·9	1·1	85·0	91	99
„ 4	7·0	1·0	90·0	98	100
„ 5	7·1	0·8	93·0	99	100
„ 6	6·9	1·1	96·0	99	100

Table IX. *Oxidation of acetic acid.*

			Purification %				
			2nd day	7th day	14th day	21st day	
			0·2 % acetic acid			0·2 % sucrose	
	p_H	$[H^·] \times 10^7$					p_H
Aspirator	4·0	1000	—	—	—	—	6·9
Section 1	4·6	250	40	36	38	44	6·6
„ 2	6·5	3·2	65	82	85	57	6·8
„ 3	6·8	1·6	87	94	97	78	6·9
„ 4	7·0	1·0	90	96	98	91	7·0
„ 5	7·1	0·8	93	96	98	98	6·9
„ 6	7·0	1·0	95	98	98	98	6·9

The reduction in $[H^·]$ is seen to be much greater in the first section than in any of the other subsequent sections, but the amount of purification effected is about equal in the first and second sections. Obviously the reduction in acidity is effected by other processes in addition to oxidation. The results show very conclusively that the development of acidity in a filter bed is not in itself detrimental to its efficiency. If anything, it appears that organic acids are more readily oxidised than sugar, a result which is not in agreement with conclusions reached by Jenkins [1931]. It follows therefore that the low purification of the fine gravel filter is not due to any possible toxic action of the organic acids, but solely to lack of oxygen. These results are also of great interest biologically since the protozoan population of sections 1 and 2 was almost entirely extinguished, so that the more efficient filtration process was carried on by bacteria and fungi supporting a population consisting of eelworms and insect larvae.

The more rapid oxidation of organic acids compared with that of sugar may have a possible explanation in terms of energy transformation.

$$C_6H_{12}O_6 = 2C_3H_6O_3 + 22{\cdot}5 \text{ kg. cals.}$$
(Glucose) (Lactic acid)

$$C_6H_{12}O_6 = 3C_2H_4O_2 + 45 \text{ kg. cals.}$$
(Acetic acid)

$$C_3H_6O_3 + 3O_2 = 3CO_2 + 3H_2O + 326 \text{ kg. cals.}$$

$$C_2H_4O_2 + 2O_2 = 2CO_2 + 2H_2O + 209 \text{ kg. cals.}$$

In the fermentation of sugar to lactic and acetic acids the organisms may derive energy for growth and metabolism without oxidising the material, but no growth is possible on lactic and acetic acids without oxidation. When sugar is supplied to a filter bed there is doubtless a development of organisms capable only of utilising intra-molecular energy, and these organisms must necessarily die out when the filter receives only organic acid, resulting in an increase in the oxidising organisms.

C. *The development of the film.*

The active portion of the filter medium consists of bacteria and fungi supporting a very variable population of protozoa, nematodes and insect larvae. It would be difficult to follow the possible fluctuations in the bacterial flora but they are doubtless reflected in the constantly changing fauna. In a filter bed the organisms develop in the form of colonies attached to the particles of the medium. On starting up a newly inoculated filter the liquid shows an increasing bacterial population as it flows through successive sections, until a certain maximum is reached about the third section, when clumping or colonisation begins, resulting in a much reduced dispersion of the bacteria in the lower sections. This process continues and gradually appears in the upper sections until all the effluents show a marked reduction in bacterial dispersion. The effect of this is shown in Table X by the results of direct chamber counts of bacteria in the effluents.

Table X.

	Bacterial numbers in millions per cc.	
Section	1st day	14th day
1	5·6	32–52
2	32	20–45
3	296	14–29
4	84	12–13
5	36	10–16
6	36	8–14

(These figures do not represent in any way the bacterial population of the film but only the free organisms in suspension. The numbers in the film vary from 3000 to 9000 millions per g.) As the fixed colonies proliferate they may become too heavy for the point of attachment and thus break away either as a whole or in portions. These small fragments of film are carried along with the liquid, provided the filter bed is coarse enough to allow them to pass through, and they may be seen in the effluent from all sections after the first or second week of operation. Under the microscope they appear as masses

of micrococci in the form of zoogloea with occasional yeast cells and funga hyphae. Frequently they carry with them large numbers of protozoa and eelworms. The movements of the larger eelworms and annelids may also play a part in the breaking away of the colony. The colour of the film is at first white especially in the upper sections, but after 3 weeks it changes gradually to a dark brown in the lower sections. This shedding of film is a constant process in the life of the filter and is quite distinct from the periodical sloughing which is associated with seasonal climatic changes.

The rate of growth of the film will be greatest in the first section where the concentration of food is greatest and will diminish with the depth of the section. This explains the greater purification effected in the upper sections of the filter bed. According to Penfold and Norris [1912] the time of generation of *B. typhosus* in peptone increases from 60 minutes at 0·2 % concentration to 90 minutes at 0·1 % and 180 minutes at 0·05 %. A similar result was obtained by Cutler and Crump [1923] for protozoa, the concentration of the nutrient in this case being expressed in terms of bacterial numbers. With a purification of 75 % in section 3 the concentration of nutrient in this section would correspond to 0·05 % sugar and, assuming the time of generation in mixed culture to be similar to that in pure cultures, it would be about three times that of the first section, so that the film would only grow at one-third the rate. Similarly the growth rate in the fourth, fifth and sixth sections would be reduced almost to nil. The figures given in Table IV however show much greater amount of film than would be expected from these growth rates. This difference from expectation must be attributed to the film continuously shed from the upper sections being retained by the fine gravel in the lower sections. The use of a coarse medium would allow an easier passage to this discharged film. The effect of this is seen in the reconstructed gravel filter. When the first three sections were filled with coarse gravel the discharge of film into the surface of section 4 (fine gravel) was so great that it became covered in a period of 12 weeks with a solid layer $\frac{1}{2}$ inch thick.

D. *Time of contact.*

The development of the film has an effect on the filter apart from its increase in population, *viz.* in increasing the "time of contact" or the length of time the liquid takes to pass through the filter. This factor is usually determined by Clifford's method [1907] which measures the mean time required to wash out a quantity of sodium chloride from the filter at a given rate of flow.

It is obvious that the time of contact will increase with the development of the film. The film consists of about 97 % by weight of water and the total volume in one of these experimental filters may be $1\frac{1}{2}$ litres. With a rate of flow of 5 litres per 24 hours the water of the film can be changed $5/\frac{3}{2}$ times in 24 hours which gives a time of 7·2 hours for each change of water.

The volume of film in each section is measured by the difference between

the volume of the interstitial space of the clean gravel, and that after development of the film. This determination was carried out on one of the sectional filters filled with gravel of $\frac{1}{2}$ to $\frac{3}{4}$ inch after running 1 month. The results are given in Table XI. The time of contact is the ratio $\frac{\text{volume of film}}{\text{rate of flow}}$, since if X = volume of film and V = total volume of liquid per 24 hours, then V/X = number of times the film is filled per day, and $\frac{24}{V/X}$ = time of contact = $X\,24/V$.

Table XI.

Section	Volume of film cc.	Rate of flow	Time of contact hrs.
1	450	5 litres per day	2·1
2	400		2·0
3	300		1·5
4	150		0·7
5	75		0·36
6	75		0·36

From the above table we find that out of a total of 7 hours of contact 5·6 hours are spent in the first three sections. The time of contact is greatest where nutrition and growth are greatest. By reducing the size of the gravel, thereby causing a retention of the film, the time of contact can be increased considerably but not without diminishing the aeration, and increased contact without aeration is of no value. Increase in the time of contact should be the result of growth rather than mechanical accumulation. A deficiency in time of contact only occurs when starting up a new filter, and an excess tends to occur with age. This initial deficiency can be best remedied by increasing the concentration of the nutrient solution thereby increasing the rate of growth of the film. If at the same time the rate of flow is diminished, the capacity of the filter will not be exceeded. As the film develops the concentration of the solution can be diminished and the rate of flow increased.

E. *Rate of flow.*

It has been shown that the development of film in a filter is greatest in the upper sections and that it is determined by the concentration of nutrients in the liquid. Increase in the rate of flow will diminish the time of contact. This will result in a lower purification gradient, which means a higher concentration of nutrient in the lower sections, and an increase in the development of film below the upper layer. Thus with a lower purification gradient a deeper filter becomes necessary.

A problem of long standing to the sewage engineer is the question whether it is better to filter a given amount of organic matter as a strong solution at a slow rate or a dilute solution at a quicker rate. Information on this point can be obtained from a consideration of the previous figures for the sectional purification. In Table VI by averaging the figures for the normal working periods, the 2nd, 4th, 6th, 9th, 13th and 15th week, we get the following percentage purification: section 1, 64 %; section 2, 82 %; section 3, 88 %.

Since the rate of flow through five sections is 100 g.y.d. the rate of flow through each section alone is five times as much or 500 g.y.d. A 0·2 % sucrose solution run at this rate undergoes a purification of 64 %, whilst the effluent of this section containing the equivalent of 0·072 % sucrose, run through the second section at the same rate, undergoes a purification of 18 % of 0·2 = 0·036 or 50 % and the effluent of section 2 containing the equivalent of 0·036 % sucrose undergoes a purification of only 6 % of 0·2 = 0·012 or 33 %.

Thus a reduction in the concentration of nutrient through a filter diminishes its efficiency, though the purity of the final effluent is increased. The effect of increasing the rate of flow with a reduction in concentration of sucrose was studied by means of two sectional filters filled with the same gravel, $\frac{1}{2}$ to $\frac{3}{4}$ inch. Both were inoculated with the same medium. One was run with a 0·2 % solution of sugar at 100 g.y.d. and the other with 0·1 % solution at 200 g.y.d. The experiment was run for 30 days after which the gravel was washed and the amount of film determined in each section.

The results of the purification are given in Table XII.

Table XII.

	Number of days							
	6		13		20		30*	
Section	0·2 %	0·1 %	0·2 %	0·1 %	0·2 %	0·1 %	0·2 %	0·1 %
	Purification %							
3	90	59	90	72	88	74	72	50
6	96	90	99	98	99	97	97	83

* Nitrogen omitted from both filters.

There was little difference between the final purification in both filters until nitrogen was omitted from the solution. But throughout the experiment the purification at section 3 was distinctly in favour of the more concentrated solution. The effect of dilution is not apparent with a filter 6 feet deep but with a shallow filter of only 3 feet the use of a more dilute liquid at increased rate of flow gives a lower purification.

The weights of film on the various sections are given in Table XIII.

Table XIII. *Distribution of film with different concentrations of sucrose.*

	0·2 % solution at 100 g.y.d.		0·1 % solution at 200 g.y.d.	
Section	Total film (g.)	% ash	Total film (g.)	% ash
1	16·5	26	15·8	26
2	16·0	32	14·9	33
3	10·4	37	12·5	38
4	6·0	42	7·8	44
5	4·3	44	5·6	57
6	4·1	46	4·5	59
Total	57·3		61·1	

It would seem that the amount of film is greater in the upper sections and diminishes more rapidly with the 0·2 % solution at 100 g.y.d. than with the

0·1 % solution at 200 g.y.d. With the more rapid rate of flow the amount of film is greater in the lower sections, part of this increase being due to a greater ash content.

F. *The influence of the C/N ratio on the film.*

The effect of omitting nitrogen from the sugar solution has been shown in the purification figures in Tables V and XII. Most of the previous work on biological filtration has been done with liquids of a high nitrogen content and having a C/N ratio less than 10. The pulp-press liquor from a beet sugar factory has a C/N ratio of about 20, but since most of the nitrogen exists as organic nitrogen it is not immediately available for the nutrition of the organisms of the filter. Such a liquor would thus resemble a liquid with a higher C/N ratio. The growth of the film in the filter bed has a certain definite nitrogen requirement without which its development will be retarded. An analysis of the film from the top and bottom sections of the gravel filter (see Table IV), is given in Table XIV.

Table XIV. *Nitrogen content of film.*

Section	Total nitrogen (Kjeldahl)	% organic matter	% nitrogen in organic matter
1	7·1	67·0	10·6 = 69 % protein
6	4·6	38·7	13·1 = 85 % protein

The percentage of nitrogen in the organic matter is high, especially in the film of section 6 which contains nitrifying organisms. It is equivalent to a protein content of 85 % so that the organisms of the lowest section have a very low non-protein content (15 %) similar to that of animals in a state of starvation. Probably they are living on their own tissues since there is practically no soluble organic matter in the liquid passing through this portion of the filter.

A C/N ratio of 20 is equivalent to a nitrogen content of 2·1 % of the weight of sugar. In order to increase this nitrogen content to that of the film in the upper section, *viz.* 10·6 %, 4/5 of the sugar must be oxidised to CO_2. The remaining 1/5 of the sugar is then just sufficient to combine with the 2·1 % of nitrogen to form the organic matter of the film. If more than 4/5 of the sugar were oxidised there would not be sufficient to combine with all the nitrogen, and the excess nitrogen would be excreted. Thus, the nitrogen requirements of the film can be measured by adding the nitrogen as ammonium sulphate and noting its rate of absorption in the various sections of the filter.

For this purpose four filters were run on 0·2 % sugar solutions containing different amounts of ammonium sulphate, so as to give C/N ratios of 5, 10, 15 and 20 respectively. The effluents from the various sections were tested for ammonia with Nessler's reagent. A large excess of ammonia occurred in all sections of the filters with ratios of 5 and 10. In the filter with a ratio of 15 the excess ammonia was very slight in sections 5 and 6, whereas with a ratio of 20 no ammonia was found in sections 4, 5 and 6. It appears therefore that the limit of the nitrogen requirements of the filter medium lies between the

ratios of 15 and 20, which is in agreement with the oxidation of 85 % of the sugar and the conversion of 15 % into organic matter of the film.

To test the possibility of nitrogen passing through the filter as amino-acid, formaldehyde titrations were carried out on the effluent from sections 2 and 3. The results are given in the following table.

Table XV. *Formaldehyde titration values of filter effluents.*

	C/N ratio			
Sections	5	10	15	20
	cc. of *N*/10 NaOH required per 100 cc. of effluent			
2	4·3	1·8	1·1	0·1
3	3·9	1·7	1·0	0·1

The titration value is negligible except in the case of a C/N ratio of 5. A titration value of 4 cc. is equivalent to 0·0056 % amino-nitrogen or 1/3 of the nitrogen supplied as ammonium sulphate. It must be concluded that amino-nitrogen is not produced in excess of the requirements of the organisms except in the case of a low C/N ratio. Similar determinations on the effluents of the fine gravel filter showed an increase in the formaldehyde titration value from 2·0 to 3·7 when ponding occurred, which suggests that the formation of amino-acid in the filters was due to some disturbing factors: in the one case possibly excess of NH_4 ions (ammonium sulphate), and in the other lack of aeration. The absence of ammonia from the effluents of the filter with a C/N ratio of 20 can therefore only be due to nitrogen shortage.

The percentage purification results obtained with the various C/N ratios are given in Table XVI.

Table XVI. *Percentage purification.*

		C/N ratios			
Days	Section	5	10	15	20
2	6	83	86	79	78
7	6	93	91	92	83
14	1	41	36	30	32
	2	68	60	71	58
	3	91	73	88	76
	4	94	91	89	84
	5	95	92	90	90
	6	95	92	92	92
21	1	58	59	56	53
	2	74	76	75	72
	3	95	82	85	86
	4	98	97	94	92
	5	99	98	97	96
	6	99	98	98	98
28	1	18	28	45	43
	2	42	57	51	61
	3	60	67	83	73
	4	81	71	87	94
	5	91	90	90	96
	6	97	96	96	97

It will be seen that in the early stage of the development of the film a low C/N ratio gives a better purification, but this difference does not persist beyond 14 days. On the 21st day there is practically no difference between the various filters as regards purification in any of the sections. On the 28th day the filters with the low C/N ratio show a distinct falling off in purification in the first three sections, but there is no significant difference between the final effluents.

The filters were dismantled after 30 days and the amounts of film on each section determined. The results are given in Table XVII.

Table XVII. *Effect of C/N ratio on dry matter of film.*

	C/N ratio							
	5		10		15		20	
Section	Film	Ash	Film	Ash	Film	Ash	Film	Ash
	g.	%	g.	%	g.	%	g.	%
1	18·7	21	18·6	20	16·8	25	17·1	22
2	17·9	25	17·7	27	16·4	31	15·3	27
3	12·8	33	11·8	34	10·4	37	9·7	37
4	9·7	40	9·7	40	6·4	42	7·0	43
5	4·8	56	5·0	62	4·3	44	6·6	62
6	4·1	59	4·2	63	4·1	51	4·0	63
Total	72·3		71·0		58·4		63·3	

Total weight of sugar consumed per filter = 300 g.

Deducting the ash content of the film we obtain a total weight of film produced of 50·0 g. from the C/N 5 filter and 39·2 g. from the C/N 15 filter, representing 17 % and 13 % respectively of the weight of sugar consumed. This leaves 83 % and 87 % to be accounted for by the sugar oxidised and that remaining as impurity in the final effluent. These results are rather better than those obtained in the 5-day test (p. 1421), but inferior to the 90 % obtained by Cutler and Crump [1923] in the pure cultures with continuous aeration.

From these results it would appear that the better purification obtained in the early stages with a low C/N ratio (5–10) is due to a more rapid growth of film, which may later become excessive and diminish aeration with consequent reduction of efficiency.

Some control over the growth of film would appear to be desirable for the maintenance of efficiency. Of the various limiting factors to bacterial growth, two only appear capable of application in practice, *viz.* (1) dilution, (2) reduction of nitrogen (only applicable where nitrogen has been added). Direct biological control of the population has not yet been attempted, but experiments in this direction would doubtless supply useful information.

A reduction in growth rate of the organisms can be effected by diminution in the nitrogen supply to a C/N ratio of 100. This occurred when ammonium sulphate was omitted from the solution, but in such cases there was an immediate drop in the purification. It must therefore be concluded that a certain growth rate is essential for efficient filtration.

The maintenance of mature organisms with a high CO_2 output does not appear a possible solution. The most hopeful line of biological control of the film would appear to be in the use of a metazoan population to effect a more rapid consumption and discharge of film (*cf.* acetic acid experiment, p. 1430).

G. *Nitrogen fixation.*

Microscopic examination of the effluents of the various sections revealed the presence of organisms resembling the capsular and sarcina forms of *Azotobacter*, the nitrogen-fixing organism. Various workers on filtration have suggested the possibility of nitrogen fixation taking part in the processes of the filter bed. A sectional filter was therefore run for 14 days on 0·2 % sugar solution *plus* mineral salts but without added nitrogen. The tap water used for dilution contained 8 parts of nitrate-nitrogen per million, equivalent to a C/N ratio of 100. The gravel was washed before commencing the trial and a small inoculation of the previous film was used. To assist the establishment of a film 0·006 % nitrogen as ammonium sulphate was added to the first day's charge in the aspirator.

The purification figures and weights of film obtained are given in Tables XVIII and XIX.

Table XVIII. *Purification of nitrogen-free solution.*

	Percentage purification	
Section	After 4 days	After 14 days
3	52	58
6	65·5	76

Table XIX. *Weights of film from nitrogen-free solution.*

	Weight in g.		Total nitrogen
Section	Total	% ash	%
1	17·6	8·1	
2	10·1	11·0	2·02
3	12·1	14·5	
4	7·7	21·0	
5	6·9	34·0	1·46
6	5·9	35·0	

Total nitrogen of the film (Table XIX):

$$\left.\begin{aligned}\text{From sections 1, 2 and 3} \quad & 39{\cdot}8 \times \frac{2{\cdot}02}{100} = 0{\cdot}8039 \\ \text{From sections 4, 5 and 6} \quad & 20{\cdot}5 \times \frac{1{\cdot}46}{100} = 0{\cdot}2993\end{aligned}\right\} = 1{\cdot}1032.$$

Nitrogen supplied in solution:

$$\left.\begin{aligned}5 \text{ litres} \times 0{\cdot}00006 \text{ g. N per cc.} &= 0{\cdot}3 \text{ g.} \\ 13 \times 5 \text{ litres} \times 0{\cdot}000008 \text{ g.} &= 0{\cdot}52 \text{ g.}\end{aligned}\right\} = 0{\cdot}82$$

Gain in nitrogen = 0·2832 g.

Sugar passed through filter

$$14 \times 5 \text{ litres} \times 0{\cdot}002 = 140 \text{ g.}$$

Assuming 70 % consumption of sugar by the organisms

Sugar consumed = 98 g.

Ratio of sugar consumed/nitrogen fixed $= \frac{98}{0{\cdot}2832} = 345.$

These results show a small but definite gain in nitrogen similar in amount to that obtained by various workers on nitrogen fixation. The effluent contained no mineral nitrogen but probably contained a small amount of organic nitrogen in the form of living organisms, so that the total fixation of nitrogen was no doubt greater than the above figures show.

From the point of view of biological filtration nitrogen fixation cannot be said to be of any real importance. With a C/N ratio of 100 the amount of nitrogen fixed is only adequate to affect a purification of 76 % after 2 weeks' running with a 6 ft. filter. Assuming the same increase in purification with depth as in previous experiments it would require a filter at least 12 ft. deep to effect a purification of 98 % using a solution with a C/N ratio of 100.

H. *Nitrification.*

The production of nitrates in a filter effluent has always been a criterion of purification. In the experiments with sectional filters the effluents from each section were tested daily for nitrite by the Griess-Ilosvay reagent. In starting up a new filter with 0·2 % sugar a period of 20 days may elapse before any nitrite appears in the effluent. The effluents from the solutions of C/N ratio 5 and 10 showed the presence of nitrite several days before the effluent from a C/N ratio of 15 or 20, and the amount of nitrite was always greater from the solution of low C/N ratio.

The amount of nitrite produced diminishes from section 6 upwards to section 4, but none is found in the upper sections irrespective of the C/N ratio. The presence of nitrifying organisms in these upper sections has been shown by applying the Griess-Ilosvay test to the dilution water after incubation in the 5-day test. These sections therefore provide an example of the suppression of nitrification even with a C/N ratio of less than 5. It would appear therefore that the degree of purification has a greater influence on nitrite formation than has the C/N ratio [*cf.* Jensen, 1929]. When this figure reaches 85 to 90 %, *i.e.* equivalent to not more than 0·03 % sugar, nitrification may appear. It has been shown that bacterial growth is much more rapid in the upper sections, being 6 times more rapid in section 1 than in section 4. This growth would have a large oxygen requirement which would be met by the reduction of any available reducible substance. In these circumstances nitrous oxide could not persist. In sections 4, 5 and 6 aeration is generally more than sufficient to maintain the oxygen requirements of the organisms. For these reasons nitrite formation only persists during the final stages of purification.

The presence of oxidisable carbohydrates does not in itself necessarily inhibit nitrification. Nelson [1931] has recently shown that *Nitrosomonas* in

pure culture may continue to oxidise ammonia even in the presence of 10 % glucose.

In the case of the filters run on lactic and acetic acids the rate of purification was more rapid and strong nitrification appeared in section 3.

Certain pure strains of bacteria isolated from the filter bed at Colwick appeared to be capable of oxidising ammonium lactate to CO_2 and nitrous acid simultaneously, but in the presence of restricted aeration the nitrite did not persist [Rothamsted, 1929].

In the case of the clinker filter supplied with a solution of C/N ratio 15, the amount of nitrite in the final effluent reached 3 parts per million on the 30th day, and increased to a maximum of 35 parts on the 50th day. With the increase in nitrification all trace of ammonia disappears from these sections. The maximum amount of nitrite-nitrogen obtained in this experiment is slightly more than half the total amount supplied to the filter as ammonia. This means that nearly half the nitrogen added with the sugar is retained in the filter as organic matter and is only slowly released by decomposition.

Considerable fluctuations in the amount of nitrite were recorded from time to time, but they were generally traceable to irregularities in the rate of flow or mechanical stoppages resulting in deficient aeration. In the fine gravel filter nitrification steadily declined after the 30th day as a result of the diminishing purification of this filter. After the regrading of the upper three sections nitrification reappeared in the lower sections and continued vigorously.

The production of nitrates was detected 10 days after the appearance of nitrites, but it developed more slowly, apparently requiring the stimulus of larger amounts of nitrite. Increase in the amount of nitrate occurred at the expense of the nitrite, until all trace of nitrite disappeared from sections 5 and 6 and only a small amount remained in section 4. The amounts of nitrate in the final effluent varied considerably from time to time from 1 part N to as much as 10 parts N per 100,000, which is more than the rate of supply of nitrogen to the filter. To some extent these variations were due to differences in aeration but not entirely. The fact that they were of a much higher order than occurred in the previous period of nitrite formation suggests that the variation is of a biological nature. Fluctuations in the C/N ratio of the original solution had no direct effect on the amounts of nitrate in the effluent.

To obtain further information on this point, section 6 of the filter was removed and treated as a separate filter. Solutions containing nitrogen in different forms and amounts were run through this section at the rate of 2 litres per day (Table XX). The solution of each substance was run through the filter for not less than 24 hours. Estimations of the three forms of mineral nitrogen were made by the colorimetric methods as described in the Ministry of Health Bulletin [1929], ammonia by Nessler's reagent, nitrite by Griess-Ilosvay reagent, and nitrate by the pyrogallol test.

Table XX.

All quantities expressed in parts per million of solution.

Source of nitrogen	Total N	Amount of N in effluent as: Ammonia	Nitrite	Nitrate	Gain or loss	
Ammonium lactate	200	40	4	120	—	−36
,,	150	100	8	44	+ 2	—
Nil. Water only	—	—	0·5	40	+ 40	—
,,	—	—	1·0	24	+ 25	—
Ammonium lactate	60	40	1·0	4	—	−15
,,	60	30	0·2	32	+ 2	—
Urea	30	1	0·2	16	—	−13
,,	60	10	0·2	13	—	−37
Nil. Water only	—	—	—	12	+ 12	—
Ammonium sulphate	140	40	1	100	+ 1	—
,,	60	50	—	16	+ 6	—
Sodium nitrite	40	—	15	40	+ 15	—
,,	20	—	0·2	40	+ 20	—
,,	60	—	18	120	+ 78	—
Nil. Water only	—	—	—	20	+ 20	—
Sodium nitrite	60	—	8	80	+ 28	—
,,	100	—	15	120	+ 35	—
,,	100	—	0·2	200	+100	—
,,	100	—	10	160	+ 70	—
,,	100	—	0·2	160	+ 60	—
,,	180	—	48	320	+188	—
Nil. Water only	—	—	—	32	+ 32	—
,,	—	—	—	24	+ 24	—
Sodium nitrite	100	—	16	160	+ 76	—

From these results it appears that the only nitrogenous substance capable of stimulating nitrate formation is sodium nitrite, which also gives rise to more nitrate-nitrogen than is supplied as nitrite. The increased nitrogen excreted in presence of nitrite is more than can be accounted for by the normal oxidation of the nitrogen in the film, since water alone extracts much smaller amounts of nitrate-nitrogen. It is suggested that the toxicity of the nitrite exerts some selective action on the mixed bacterial flora of the filter, resulting in increased breakdown and oxidation of the film. The symbiosis between these ammonifying and nitrifying bacteria is the subject of detailed bacteriological investigation.

J. *Phosphate metabolism of the film.*

In the preceding experiments the mineral salts added to the sugar solution included 0·05 % potassium phosphate (p. 1424). The amounts of available phosphoric acid in each effluent of the sections was estimated by the colorimetric method of Fiske and Subbarrow [1925].

The results are plotted in curve A, Fig. 6, the amounts of phosphoric acid found in the various effluents being expressed as percentages of that supplied in the sugar solution.

As would be expected the absorption of phosphate is confined to the first three sections or the region of growth of the filters. In the 6th section there is a slight increase in the amount of soluble phosphate in the effluent probably due to the oxidation of organic phosphorus and the nitrification process.

These changes in phosphate content of the effluents may be correlated with changes in the p_H of the sectional effluents. In Figs. 1 and 2 it has been shown that the p_H of sections 4 and 5 tends to increase above that of the original solution, whilst in section 6 it tends to become less. The absorption of phosphoric acid by the growing film in sections 1 to 3 would result in the formation of free potash which would combine with any organic acid produced by the decomposition of the sugar. Oxidation of these salts would then result in the formation of potassium carbonate and a consequent rise in p_H. In section 6 the oxidation of organic phosphorus and nitrification would result in a lower p_H in this section.

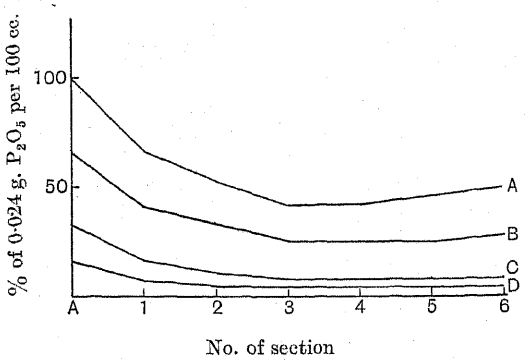


Fig. 6. Absorption curves for phosphoric acid in sectional filter.

Attempts to measure the phosphate requirements of the film by the method employed in the case of nitrogen were not successful. Reducing the amount of phosphate in the original solution even to 1/6 the amount did not result in complete absorption of the phosphate by the film (see Curves B, C and D, Fig. 6). Each reduction in phosphate supply merely diminished the phosphate gradient down the various sections. This phenomenon is probably due to the division of the phosphate between the demand for nutrition or tissue formation and the temporary requirement of the enzyme reactions involving the formation of hexosephosphate. The hydrolysis of the hexosephosphate by the mineral acid used in the colorimetric determination would account for the apparent presence of uncombined phosphate during phosphate starvation.

The effect of phosphate deficiency on the working of the filter was determined as follows.

The five sectional filters were washed and re-inoculated with equal amounts of wash water. They were then run for 3 weeks on a basic solution of 0·2 % sugar and 0·03 % ammonium sulphate to which was added potassium phosphate in different amounts as shown in Table XXI.

The highest purification figures were given by filter C receiving 0·008 % P_2O_5. Increasing the amount of phosphoric acid did not improve the filtration

Table XXI. *Percentage purification at section 3 with different amounts of phosphates in the crude liquor.*

Filter ...	A	B	C	D	E
P_2O_5 % ...	Nil*	0·004	0·008	0·016	0·024
Days of run					
2	55	57	68	64	64
8	60	65	75	73	75
15	54	74	95	95	90
21	51	75	74	72	70

* The tap water used in this experiment contained 0·08 part P_2O_5 per million.

though Fig. 6 shows that it results in an increased retention of phosphoric acid by the film. This probably indicates a greater growth of film and since it is not accompanied by a corresponding increase in purification it must be assumed that aeration has become the limiting factor. This would explain the falling off in efficiency after the 15th day.

A smaller amount of phosphoric acid than 0·008 % appears to delay the development of the film, and in the almost complete absence of phosphate the film is permanently reduced in oxidising power.

Conclusions.

Biological filtration consists of two processes, *viz.* (1) deposition of organic matter by growth of film; and (2) oxidation through respiration. Both processes must be provided for, and since they are dependent upon one another the dominating factor in a filter bed is aeration, which is determined initially by the interstitial space and subsequently by the growth of the film. In this connection it should be realised that the minimum size of the particles is the determining factor and not the average size.

The growth of film is an essential part of the process in the filter, and any condition which delays or prevents it has an adverse effect on the purification. The most important factors influencing this growth are the concentration of food material or organic matter in the solution and its composition. Both these factors are more prominent in the initial stages of the working of the filter and their effect diminishes with age. Similarly their effect is greatest in the upper layers of the bed and diminishes with depth. An adequate supply of nitrogen and phosphorus is essential to the growth of the film and when the C/N ratio is more than 15 and the C/P_2O_5 ratio more than 10 the development and efficiency of the film is reduced. The lower portion of the filter bed then becomes dependent on nitrogen fixation and the discharge of organic nitrogen and phosphorus from the upper layer. In the early stages both these sources of nitrogen are inadequate and consequently delayed purification results. This effect of a high C/N ratio is probably one of the chief difficulties in the treatment of sugar beet factory effluents.

Physiologically a satisfactory filter bed is characterised by the absence of putrefaction (*i.e.* the production and accumulation of readily decomposed

organic compounds). (See Table XV.) The energy of decomposition is consumed in growth so that the occurrence of dead organisms is only a momentary phase in the metabolism of the film. Considering the enormous protozoan population, the occurrence of dead organisms is very rare. Under anaerobic conditions however decomposition products may accumulate and result in a change in the p_H of the medium and a higher formaldehyde titration value.

The clumping and colonisation of the organisms in the bed of the filter is also a very characteristic feature. It results in a much lower bacterial population in dispersion through the solutions than would normally occur in a liquid culture. This clumping or agglutinating process appears to be connected with the degree of aeration. The effluents from the fine gravel filter were generally opalescent and passed through a filter paper with difficulty, whilst those from the coarse filters were clear and filtered through paper readily. The degree of opalescence diminishes with the depth of the filters.

Nitrification is an essential feature of an efficient filter bed since oxidised nitrogen provides the most effective hydrogen acceptor in biological oxidation.

Nitrite formation occurs when the concentration of sucrose and its derivatives in solution falls below 0·03 %. The absence of nitrification in the presence of a higher concentration of soluble organic matter does not imply the absence of the nitrifying organisms but rather that the potential rate of decomposition of the nitrite is more rapid than its production. The production of nitrite ultimately gives rise to the production of nitrate, and this process increases until nitrite formation is hardly detectable.

Summary.

1. There are two limitations to the 5-day oxygen absorption test, *viz.* (*a*) it is not possible to estimate carbohydrate in terms of oxygen absorption by a biological process owing to organic synthesis being an essential part of the process; (*b*) the nature of the inoculum affects the relative amounts of oxidation and synthesis.

Recognising the above limitations, the 5-day test is the most satisfactory measure of pollution available.

2. The production of organic acids is a stage in the decomposition of sugar, but their rapid destruction is a property of an efficient filter bed.

3. The organic acids lactic and acetic are more rapidly oxidised than sucrose.

4. The formation of oxidised nitrogen in the presence of organic matter promotes the dehydrogenation (oxidation) of lactic acid and probably of other compounds.

5. The growth of film is an essential part of the purification process and the film itself accounts for 15 % of the sucrose supplied.

6. Aeration is the dominant factor in biological filtration and is determined by the minimum size of the material which provides the interstitial

space for the growth of the film and also for its discharge. A fine medium should not be used to secure time of contact.

7. If grading of the medium is desired, the smaller particles should be placed at the bottom where the smallest amount of film will develop, but to ensure free passage of discharged film nothing passing through $\frac{1}{2}$ inch mesh should be used.

8. The rate of flow is the best means of controlling the time of contact and purification. Within limits a slow rate of flow with high concentration will increase the time of contact more rapidly than the reverse conditions. As the time of contact increases, the rate of flow may be increased without reduction in purification.

9. The growth and efficiency of film is dependent upon definite nitrogen and phosphorus requirements, *viz.* a C/N ratio of 15 and C/P_2O_5 ratio of 10.

10. Nitrogen fixation occurs in the filter bed but results in the growth of film of low N content and low purification.

11. A low C/N ratio and a high concentration of organic matter promote the rapid development of the film and attainment of high purification. They also lead to excessive growth of the film and where possible should be used only in the first month of the working of the filter.

12. With a C/N ratio of 15 or less a filter bed 5 ft. deep will give complete purification of 0·2 % sugar solution at a rate of flow of 100 g.y.d. using coarse gravel or clinker. With a C/N ratio of 50 to 100 a much deeper filter would be required, and better results could more easily be secured by the addition of nitrogen than by deepening the filter.

13. Both stages of nitrification occur in the filter bed and are subject to considerable fluctuations not correlated with the supply of nitrogen in solution.

This subject is also being investigated from the biological standpoint by Mr D. Ward Cutler, Head of the Department, Miss L. M. Crump, and Miss A. Dixon, to whom the author wishes to express his great indebtedness for constant help in consultation and practice; also to Mr E. Hannaford Richards and the members of the Fermentation Department, and to Mr Warren of the Chemical Department, for the Kjeldahl determinations.

The investigation described in this paper was carried out at Rothamsted Experimental Station as part of the programme of the Water Pollution Research Board of the Department of Scientific and Industrial Research and is published by permission of the Department.

REFERENCES.

Clifford (1907). *J. Soc. Chem. Ind.* **26**, 739.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Cutler and Crump (1923). *Biochem. J.* **17**, 878.
Fiske and Subbarrow (1925). *J. Biol. Chem.* **66**, 375.
Green and Ampt (1912). *J. Agric. Sci.* **5**, 21.
Jenkins (1931). *Biochem. J.* **25**, 147.
Jensen (1929). *J. Agric. Sci.* **19**, 71.
Ministry of Health (1929). Methods of chemical analysis as supplied to sewage and sewage effluents.
Nelson (1931). *Zentr. Bakt. Par.* II, **83**, 280.
Penfold and Norris (1912). *J. Hygiene*, **12**, 527.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
Rothamsted Expt. Station Report (1929). (Harpenden), p. 41.
Stephenson (1930). Bacterial metabolism. (Longmans, London.)
Water Pollution Research Board Report (1929). Dept. of Scientific and Industrial Research.

CLV. EFFECT OF BACTERIOPHAGE ON OXIDATION-REDUCTION POTENTIALS OF *B. DYSENTERIAE* (SHIGA) CULTURES.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

(*Received June 12th, 1931.*)

It has been shown in previous communications that the oxidation-reduction potential curves of peroxide-forming bacteria are entirely different from those of catalase-containing organisms [Hewitt, 1930]. Furthermore, when catalase is added to cultures of peroxide-forming bacteria the oxidation-reduction potential curves obtained are similar in many respects to those of organisms which themselves form catalase [1931, 1]. The behaviour of *B. dysenteriae* (Shiga) is of interest in this connection since it neither forms peroxide nor contains catalase [McLeod, 1930]. It is, therefore, of importance to determine in what respects the oxidation-reduction potentials developed in cultures of *B. dysenteriae* differ from those developed in cultures of peroxide-forming bacteria on the one hand and catalase-containing bacteria on the other.

In addition to these points the opportunity occurs with *B. dysenteriae* to study the effect of the bacteriophage phenomenon on oxidation-reduction potentials. It appeared possible that the destruction and lysis of bacteria by the action of bacteriophage might be accompanied by characteristic changes in the oxidation-reduction potentials of the cultures.

With these several objects in view observations have been made of the potentials developed in *B. dysenteriae* (Shiga) cultures and of the effect of bacteriophage.

Methods.

The methods and apparatus used were those described previously [Hewitt, 1930; 1931, 2]. In each experiment 8 cc. of culture medium were inoculated with 0·1 cc. of a 24-hour broth culture of the rough variant of *B. dysenteriae* (Shiga) and the potentials developed were followed during the active growth of the culture. The author is indebted to Dr C. Todd for a sample of Shiga phage.

Results.

Potentials of cultures without phage.

Plain broth cultures (Fig. 1). In stationary aerobic peptone-infusion broth cultures the potential fell slowly for the first 2 hours (lag period) and more

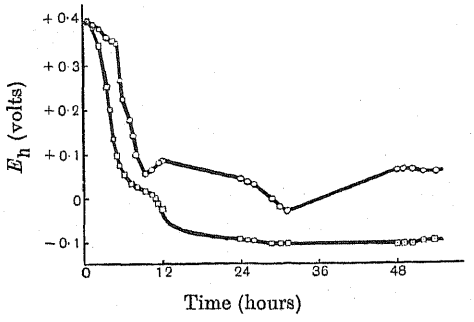


Fig. 1. Infusion broth cultures of *B. dysenteriae* (Shiga).

rapidly for the next 2 hours. The lowest level of potential (− 0·1 v.) was reached after 48 hours' growth and was maintained for several days.

The fall in potential was very sluggish also in cultures which were vigorously aerated by the method previously described [1931, 2]. After a lag period lasting some 4 hours the potential fell to the level + 0·1 v. The fall from this level to + 0·02 v. was very slow as with the stationary aerobic culture. The lowest level of potential (− 0·03 v.) was reached after 30 hours' incubation, and the potential had not risen above the level + 0·06 v. after 54 hours' growth (upper curve, Fig. 1).

B. dysenteriae (Shiga) has, therefore, feeble reducing powers and no evidence was obtained of the high levels of potential corresponding to peroxide formation. The oxidation-reduction potential behaviour resembled that of catalase-containing bacteria except that the reducing powers were less intense.

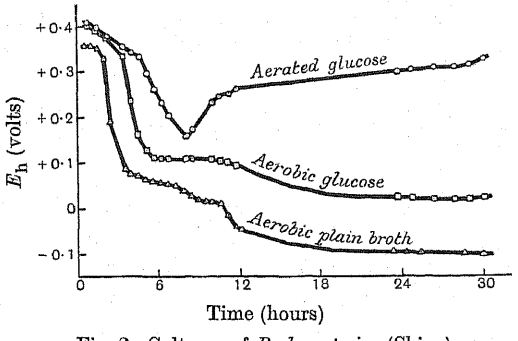


Fig. 2. Cultures of *B. dysenteriae* (Shiga).

1 % glucose broth cultures (Fig. 2). In contradistinction to the behaviour of the other bacteria investigated the potential fell much more slowly in

stationary aerobic glucose broth than in plain broth. The lowest level of potential reached was + 0·02 v. in 25 hours and the potential rose very slowly. With other organisms investigated there has been a rapid fall in potential in glucose broth followed by a rapid rise, then a slight fall and finally a more gradual rise.

In aerated glucose broth cultures the potential reached its lowest level, + 0·16 v., in 8 hours and then rose fairly rapidly. This fall in potential is intermediate between that of haemolytic streptococci (lowest level + 0·2 v.) and that of staphylococci (— 0·01 v.).

Effect of bacteriophage (Figs. 3, 4).

0·1 cc. of Shiga phage was added to an aerobic 7-hour culture of *B. dysenteriae* (Shiga) in infusion broth. After 18 hours' incubation the culture had cleared and was filtered through a small Seitz filter. The bacteriophage was subjected to several passages before it was used.

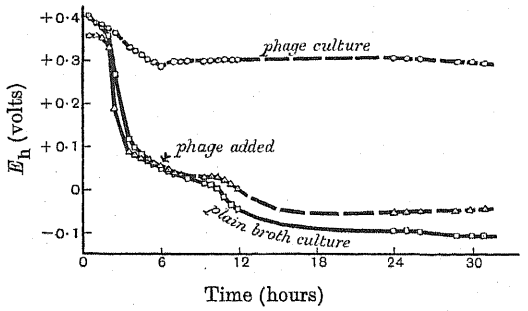


Fig. 3. Stationary aerobic cultures. (Broken lines indicate cultures containing bacteriophage.)

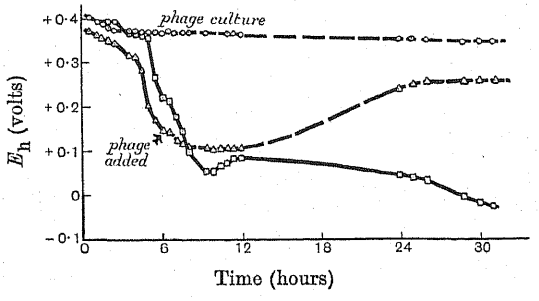


Fig. 4. Aerated broth cultures.

When the phage (0·1 cc.) was added to a culture immediately after inoculation with *B. dysenteriae* the potential fell very little even after 54 hours' incubation, and no visible growth occurred. In neither the stationary aerobic (Fig. 3) nor the aerated cultures did the potential fall below + 0·29 to 0·33 v. It is evident that the dysentery bacilli had not proliferated to any appreciable

extent and that the bacteriophage itself had no detectable effect on the potentials. The bacteriophage had merely inhibited the usual fall in potential accompanying growth of the organisms. In further experiments 0·1 cc. of bacteriophage was added to the culture after 6 hours' incubation. In these experiments the fall in potential slackened after the bacteriophage had been added. After a further 6 hours' incubation in aerated cultures (Fig. 4) and after 18 hours in stationary aerobic cultures the potential had commenced to rise. The bacteriophage appeared to have no effect other than that of inhibiting the proliferation and hence the metabolic changes and fall in potential of the cultures.

Discussion.

B. dysenteriae (Shiga) possesses only feeble reducing powers compared with other bacteria investigated. The potentials of cultures of this organism fall fairly slowly and do not reach the low levels attained by haemolytic streptococci, *C. diphtheriae*, staphylococci and pneumococci [Hewitt, 1930]. Although the Shiga bacillus does not contain catalase, it does not display the electrode potential behaviour of peroxide-forming organisms, but behaves similarly to *C. diphtheriae* and staphylococci, which contain catalase. This general similarity to the catalase-containing organisms is shown in Fig. 5.

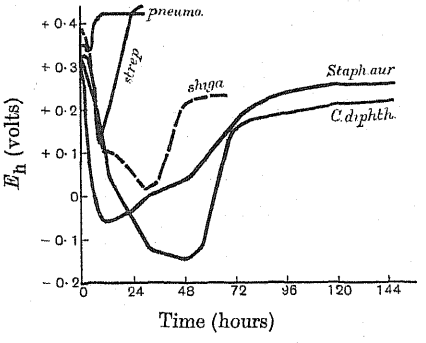


Fig. 5. Aerated broth cultures of different bacteria.

Hydrogen peroxide is formed in many biological oxidation-reduction reactions and cells of many kinds contain catalase and, hence, are able to destroy the toxic peroxide as it is formed. *C. diphtheriae* and staphylococci are examples of this type. Haemolytic streptococci and pneumococci, on the other hand, do not contain catalase and hence peroxide accumulates in aerated cultures of these bacteria. Addition of catalase to their cultures inhibits peroxide formation and certain of the characteristic features of their electrode potential behaviour disappear [Hewitt, 1931, 1]. It is remarkable therefore that the Shiga bacillus, which does not contain catalase, should not behave like a peroxide-forming organism. It is possible that the explanation of the anomaly lies in the fact that *B. dysenteriae* (Shiga) displays peroxidase activity, possibly associated with its cytochrome content [Keilin, 1929] and

thus any peroxide formed in the culture may react, in the presence of peroxidase, with broth constituents, and hence be removed as it is formed.

This is of interest from the point of view of the classification of bacteria. McLeod [1930] has classified bacteria according to their possession or non-possession of catalase and their degree of sensitivity to peroxide. As has been pointed out elsewhere [Hewitt, 1931, 2] other enzymes than catalase must affect the proliferation of bacteria under different conditions of oxygen supply, and it now seems possible that peroxidase activity may be of considerable importance in bacteria which do not contain catalase, as it may provide an alternative method of removing the toxic effects of peroxide accumulation.

Todd [1930] found that in the case of haemolytic streptococci peroxide still accumulated but in smaller amounts when peroxidase was added to the cultures. The question remains unsettled, therefore, as to whether it is the presence of peroxidase or some other difference in respiratory mechanism which accounts for the absence of peroxide formation by the Shiga bacillus.

Bacteriophage itself appears to have no effect on the oxidation-reduction potential of culture media. When added to the cultures at the time of inoculation Shiga phage inhibits the usual fall in potential accompanying the growth and metabolic activities of *B. dysenteriae* (Shiga). Thus the inhibition of growth is reflected in the lack of fall in potential. When the bacteriophage is added after the culture has grown the potential gradually ceases to fall and commences to rise as the bacteria and their metabolic activities are destroyed.

Summary.

1. *B. dysenteriae* (Shiga) possesses relatively feeble reducing powers.

2. Although it does not contain catalase its behaviour resembles that of catalase-containing organisms and not that of the peroxide-forming bacteria.

3. This behaviour may be due to peroxidase activity which is probably of some importance in the classification of bacteria.

4. Bacteriophage itself appears to have no effect on the oxidation-reduction potential but it inhibits the usual fall in potential of *B. dysenteriae* cultures by inhibiting the growth and metabolic activities of the bacteria.

The author's thanks are due to Dr C. Todd, Dr R. G. White and Dr E. W. Todd for their help and encouragement.

REFERENCES.

Hewitt (1930). *Biochem. J.* **24**, 512, 669, 676, 1551.

—— (1931, 1). *Biochem. J.* **25**, 169, 858.

—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry (London County Council).

Keilin (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.

McLeod (1930). A system of bacteriology, I, chap. VII. (Medical Research Council, London.)

Todd (1930). *Brit. J. Exp. Path.* **11**, 368.

CLVI. EFFECT OF LYSOZYME ON THE OXIDATION-REDUCTION POTENTIALS OF *M. LYSODEIKTICUS* CULTURES.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

(*Received June 23rd, 1931.*)

In a previous communication [Hewitt, 1931, 1] it is shown that the relatively slow lysis produced by adding bacteriophage to *B. dysenteriae* (Shiga) cultures inhibits the usual fall in potential accompanying the proliferation and metabolic activities of the organisms. The object of the experiments now described was to investigate the effects on the oxidation-reduction potential of the rapid lysis of *M. lysodeikticus* by lysozyme [Fleming, 1922].

Methods.

The oxidation-reduction potentials of cultures were measured by the methods previously detailed [Hewitt, 1930; 1931, 2]. For each experiment an inoculation of 0·1 cc. of a 24-hour broth culture of *M. lysodeikticus* was made into 8 cc. of culture medium. The lysozyme used in most experiments was a sterile dilution (one in ten) of egg-white in saline. In a few experiments human tears were used as a source of lysozyme; these were kindly provided by Dr F. Ridley.

Results.

Cultures without lysozyme (Fig. 1). Horse-flesh infusion broth containing 2 % of "difco" proteose peptone was used as culture medium throughout these experiments.

M. lysodeikticus gives a sparse growth in stationary aerobic broth cultures, but the growth is much more rapid and copious in aerated cultures. These effects are reflected in the corresponding oxidation-reduction potential curves (Fig. 1). In stationary aerobic cultures the potential fell slowly and did not reach the low levels which were attained by the other varieties of bacteria investigated, but in cultures which were continuously aerated by the method previously described [Hewitt, 1931, 2] the potential fell more rapidly and to

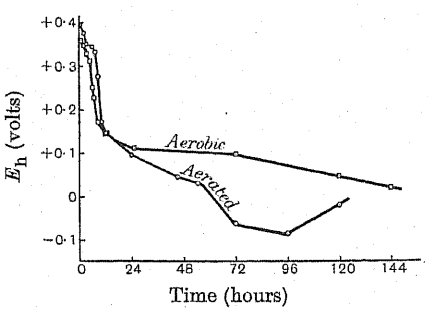


Fig. 1. Broth cultures of *M. lysodeikticus*.

a much lower level. The potentials did not at any time rise to the high levels corresponding to peroxide accumulation. This result is to be expected with an organism displaying such intense catalase activity.

Effect of lysozyme. Sterile egg-white was diluted in 9 volumes of normal saline.

Owing to the sparse growth and slow fall in potential in stationary broth cultures all experiments with lysozyme were conducted with aerated broth cultures.

Fig. 2 illustrates a typical experiment. Five cultures of *M. lysodeikticus*

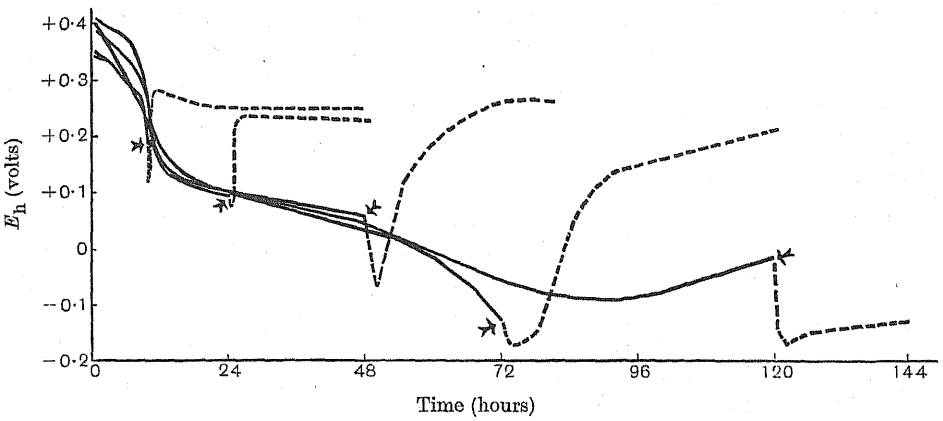


Fig. 2. Aerated broth cultures. Arrows indicate addition of lysozyme (egg-white); broken lines, cultures after addition of lysozyme; continuous lines, cultures before addition of lysozyme.

were aerated vigorously and after 9, 25, 48, 72 and 120 hours' incubation respectively amounts varying from 0·2 to 1·0 cc. of lysozyme (one-tenth egg-white) were added to the different cultures. In each case there was a sudden drop in potential when the lysozyme was added, followed by a rise in potential. The lowest level of potential was reached in each case before the culture had completely cleared and the rise in potential occurred before lysis was complete. The rapidity of the fall and subsequent rise in potential decreased with the age of culture, as can be seen from Fig. 2.

In Fig. 3 are plotted the results obtained with two cultures to which human tears were added after 48 and 72 hours respectively. A fall in potential followed by a rise before lysis was complete was observed as with egg-white, showing that lysozyme from different sources had a similar effect on the electrode potential of cultures.

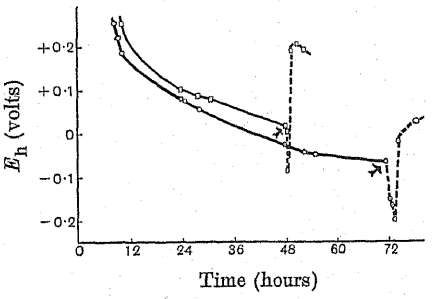


Fig. 3. Aerated cultures. Lysozyme (human tears) was added at the points marked by arrows.

When lysozyme was added during the first 25 hours' incubation (6 experiments) the average fall in potential was only 0·013 v. and on three occasions the potential during lysis did not fall below that of the original culture. In cultures to which the lysozyme was added after 29 to 120 hours' incubation (10 experiments) the average fall in potential was 0·102 v. The average time required to reach the lowest level of potential after the addition of lysozyme was 4 minutes when the lysozyme was added during the first 25 hours' incubation, and 103 minutes when added subsequently. The lowest level of potential observed in any broth culture of *M. lysodeikticus*, aerobic or aerated, was − 0·139 v. but in a lysed culture a value of − 0·205 v. was attained.

Discussion.

M. lysodeikticus displays marked catalase activity, and no evidence of peroxide formation or of the high levels of potential corresponding to peroxide accumulation was obtained. The general behaviour of the bacteria therefore resembles that of *C. diphtheriae*, *Staphylococcus aureus* and *B. dysenteriae* and not that of the haemolytic streptococcus or the pneumococcus [Hewitt, 1930; 1931, 1, 2]. *M. lysodeikticus* cultures are the only ones so far examined in which the potential falls to a lower level and more rapidly in aerated cultures than in stationary aerobic cultures. Despite the oxidising effect of the increased oxygen supply the bacteria were more easily able to establish reducing conditions when the cultures were aerated, and this was associated with the greatly increased growth under these conditions. Oxygen, therefore, has a negligible effect on the electrode potentials directly, but indirectly its presence enables this obligate aerobe to effect metabolic changes which are impossible when oxygen is absent or present only in small amounts. Evidently *M. lysodeikticus* is deficient in those enzymes which are necessary to effect oxidation-reduction reactions in the absence of air, as shown by the sparse growth and slight fall in potential when the oxygen supply is restricted. Micro-spectroscopic examination revealed that *M. lysodeikticus* like other aerobic organisms contains cytochrome and it seems therefore that this substance, although able to catalyse aerobic oxidations [Keilin, 1929], plays no part in anaerobic reactions, probably because it is fully reduced and inert in the absence of an abundant oxygen supply. Anaerobes are stated not to contain cytochrome.

Table I.

Organism	Lowest levels of potential reached in cultures: Aerobic E_1	Aerated E_2	Difference $E_2 - E_1$
Pneumococcus	−0·16 v.	+0·33 v.	+0·49 v.
Haemolytic streptococcus	−0·17	+0·12	+0·29
Staphylococcus aureus	−0·19	−0·12	+0·07
B. dysenteriae (Shiga)	−0·11	−0·04	+0·07
C. diphtheriae	−0·21	−0·15	+0·06
M. lysodeikticus	−0·00	−0·09	−0·09

In Table I are summarised the lowest levels of potential reached by different bacteria in ordinary aerobic and in continuously aerated broth cultures.

The results with pneumococcus and haemolytic streptococcus are complicated by the factor of peroxide formation, which limits the fall in potential in aerated cultures. When catalase is present in cultures of pneumococcus the difference $E_2 - E_1$ is only about 0·29 v. and that of the haemolytic streptococcus about 0·23 v. [Hewitt, 1931]. With *S. aureus* growth is more luxuriant in aerated than in stationary aerobic cultures, but the difference is much greater in the case of *M. lysodeikticus*. The oxidation-reduction potential in aerated cultures depends upon a balance between the reductions effected by the bacteria and the direct oxidation of the reducing substances by oxygen. Oxygen appears to have little effect on the potential directly but, on the one hand enables the cell to effect the reductions associated with metabolic changes, and on the other hand oxidises certain of the metabolic reduction products.

It has been shown in a previous communication [Hewitt, 1931, 1] that the slow lysis produced by the addition of bacteriophage to *B. dysenteriae* (Shiga) cultures has the effect of inhibiting the fall in potential associated with the metabolic activities of the organisms, but no other effect was observed. A further effect has, however, been observed during the rapid lysis of *M. lysodeikticus* by lysozyme in aerated cultures. Immediately lysozyme is added to a culture there is a rapid fall in potential. In sixteen experiments the fall ranged from 0 to 0·149 v. (mean 0·068 v.). The fall during the lysis of cultures less than 25 hours old is very rapid but comparatively slight, whereas in older cultures containing more bacteria the fall although less rapid is much greater. The potentials of the cultures cease to fall and commence to rise before lysis has approached completion and the rise is much more rapid in young than in old cultures. Both egg-white and human tears produced these effects. The rise in potential after lysis may reasonably be attributed to the destruction of the metabolic activities of the cell as in the case of bacteriophage, but the explanation of the fall in potential during lysis is more conjectural.

In this connection it is necessary to define current views of the mechanism of establishment of reducing conditions in bacterial cultures. Coulter and Isaacs [1929], in the case of *B. typhosus*, state that "the rôle of the bacteria is the establishment of the anaerobic state" and that "this point of view is opposed to that which regards the potentials of bacterial cultures as a manifestation of reductive substances liberated by the bacteria." It seems more reasonable, however, to assume that neither of these hypotheses is correct, but that the bacteria effect reduction as a natural result of metabolism at the cell surface. To obtain the energy necessary for proliferation, food materials in the broth must be oxidised, and for this purpose free oxygen, combined oxygen, hydrogen acceptors (or electron acceptors) and every type of possible oxidising agent may be utilised by aerobic organisms. The actual agents used by the bacteria depend on the catalysts and enzymes available, and one after

another of the oxidising systems will be reduced as the cell calls its activities into play [Hewitt, 1931, 2]. The view that bacteria can effect reductions only by (i) removal of free oxygen dissolved in the medium, and (ii) liberation of reducing substances from lysed cells, appears to have little biological significance.

Accepting the view of Coulter and Isaacs [1929] bacteria would be regarded as reservoirs of highly reduced material, and the fall in potential during the lysis of the *M. lysodeikticus* would be ascribed to the sudden liberation of highly reducing substances from the lysed bacteria.

An alternative explanation lies in the hypothesis of a temporary increase in the enzymic activity of the bacteria during the lytic process. Penrose and Quastel [1930] have shown that after lysis of *M. lysodeikticus* certain enzymic activities, such as that of catalase, are augmented, whilst others, including those of the dehydrogenases, are almost wholly destroyed. Catalase is presumably contained in the cells and when these are lysed the enzyme is liberated and comes into intimate contact with its substrate. This is in accordance with the observation [Hewitt, 1931, 1] that a freshly washed suspension of *M. lysodeikticus* reacts sluggishly with hydrogen peroxide, whereas a suspension which has been allowed to stand for some time, and probably contains some broken-down cells, gives a brisk effervescence with peroxide.

Since the dehydrogenase activity disappears when the bacteria are lysed it is assumed that this enzymic activity is dependent on the integrity of the cell structure. These results refer to totally lysed cultures, but it is possible that in cultures undergoing the process of lysis there is an intermediate condition in which there is increased enzymic activity displayed by partially lysed bacteria.

The enzymic activities might be represented by the following scheme:

	Catalase activity	Dehydrogenase activity
Before lysis	+	+ +
During lysis	+ +	+ + + +
After lysis	+ + +	±

The increased dehydrogenase activity would occur when the cells were partly broken down so that there would be intimate contact between the enzymes and substrates, but before the cell structure, upon which certain enzymic activities may be assumed to depend, is completely destroyed. It should be remembered in this connection that the fall in potential noted during lysis ceases before lysis is complete. In each experiment the potential had commenced to rise before the culture had totally cleared. This observation affords some support for the hypothesis that the partially lysed cells are capable of a temporary increase in dehydrogenase activity. This temporarily increased activity of bacterial enzymes would be accompanied by intense reducing effects and hence would be reflected in a fall in the potential.

SUMMARY.

1. *M. lysodeikticus* cultures contain catalase and afford no evidence of peroxide formation.

2. Growth is more luxuriant and the fall in potential is greater in aerated cultures than in stationary aerobic cultures.

3. *M. lysodeikticus* contains cytochrome which appears not to catalyse anaerobic oxidation-reduction reactions.

4. On addition of lysozyme (egg-white or human tears) to cultures there is a rapid fall in potential followed by a rise before lysis is complete.

5. The older the culture the slower is the fall and subsequent rise in potential when lysozyme is added.

6. The fall in potential produced by lysozyme is tentatively ascribed to an increase in the enzymic activity (*e.g.* dehydrogenase) of the partially lysed bacteria during lysis.

The author is indebted to Dr R. G. White, Dr E. W. Todd and Dr F. Ridley for their help and interest.

REFERENCES.

Coulter and Isaacs (1929). *J. Exp. Med.* **49**, 711.
Fleming (1922). *Proc. Roy. Soc. Lond.* B **93**, 306.
Hewitt (1930). *Biochem. J.* **24**, 512, 669, 676, 1551.
—— (1931, 1). *Biochem. J.* **25**, 1447.
—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry (London County Council).
Keilin (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
Penrose and Quastel (1930). *Proc. Roy. Soc. Lond.* B **107**, 168.

CLVII. ON AN ENZYME FROM BLOW-FLY LARVAE (*LUCILIA SERICATA*) WHICH DIGESTS COLLAGEN IN ALKALINE SOLUTION.

By RALPH PERCIVAL HOBSON.

From the Department of Entomology, London School of Hygiene and Tropical Medicine.

(*Received July 9th, 1931.*)

It is well known that sclero-proteins are not readily digested by the enzymes of vertebrates; both pepsin and trypsin act upon elastin, collagen is very resistant to trypsin but is digested by pepsin, and keratin is not attacked by either enzyme. However, Stankovic, Arnovlzevic and Matavulj [1929] have described a keratinase from the crops of certain birds of prey and Ssadikov [1927] has claimed that a collagenase is present in ox-pancreas and in some commercial samples of pancreatin. According to earlier workers, the pancreatic enzymes are unable to digest collagen [Sahli, 1924].

Lucilia larvae normally develop in meat or carrion, but they are also a serious pest to living sheep, feeding first in the wool and later boring into the tissues. Under suitable conditions the larvae can consume meat entirely, leaving no trace of connective tissue, and, since they cannot ingest solid particles of meat, the digestion of connective tissue must occur outside the body. The larvae secrete a protease which acts in alkaline solution and persists in the excreta [Hobson, 1931]. An investigation, therefore, was made of the action of the excreta on connective tissue proteins. Their action on keratin was also studied, as a keratinase, if present, would explain how the larvae feed in the wool of sheep and penetrate the skin.

Experimental.

The excreta were obtained by placing washed larvae in a filter and allowing water to drip slowly through the filter for several hours. The product is a dark brown alkaline liquid which consists of diluted excreta probably admixed with saliva; its reaction varies from p_H 8·2 to 8·8. Collagen was prepared from the *tendo Achillis* of an ox and from catgut, elastin from the *ligamentum nuchae* of an ox, and keratin from pure wool. In each case the soluble proteins were removed by washing with water and a brief digestion with trypsin.

The action of the excreta on sclero-proteins.

Preliminary qualitative experiments showed that the excreta dissolved small pieces of collagen and elastin completely, the solution showing an increased formaldehyde titration; no digestion, however, occurred with keratin. The relative activity of the excreta on the different proteins and the results obtained with a pancreatin solution are shown in Table I. The digestions

Table I. *The action on sclero-proteins of the excreta from* Lucilia *larvae and trypsin.*

	Excreta preparation	Pancreatin solution	
Collagen*	12	0	mg. dissolved
Elastin	16	25	
Keratin	0	0	
Gelatin	1·5	1·4	Increase in formaldehyde titration in cc.

* *Tendo Achillis.*

were carried out for 48 hours at 37° at p_H 8·0 in the presence of thymol. Proteolytic activity was determined by formaldehyde titrations of gelatin digestions and the action on sclero-proteins by weighing the residues after digestion and estimating the amounts dissolved. The results show that the excreta dissolve collagen, whereas a pancreatin solution of about the same proteolytic strength has no effect. Elastin, however, is digested more readily by pancreatic trypsin than by the protease in the excreta and neither enzyme attacks keratin. Controls, with enzyme solutions which had been heated for 5 minutes at 100°, showed no action.

Attempted separation of collagenase and protease.

Ssadikov [1927] claimed that the collagenase which he found in pancreatic extracts could be separated from the enzyme which digests fibrin (fibrinase) by adsorption with kaolin or charcoal. Kaolin adsorbed the collagenase leaving the fibrinase in solution, while with charcoal the reverse was found. However, with pancreatin solutions Ssadikov obtained different results; after treatment with kaolin the fibrinase was present in both adsorbate and filtrate, the collagenase disappearing, while with charcoal the fibrinase and collagenase were present in both fractions.

These experiments were repeated with the excreta from *Lucilia* larvae. 8 cc. portions of the excreta preparation were shaken at intervals for 30 minutes with 1 g. of the adsorbent, filtered and aliquots of the filtrate tested upon gelatin and catgut, digestion being measured by formaldehyde titration and loss of weight respectively. In addition, the stability of the enzymes in the excreta was studied by measuring the activity of samples which had been kept for different periods at 37°. The results obtained with two preparations of excreta are given in Table II. It is evident that the treatment with charcoal and with kaolin has reduced the activity towards both gelatin and collagen

Table II. *The stability and adsorption of the enzymes in the excreta of* Lucilia *larvae.*

Exp.	Enzyme and treatment	Time of digestion in hours	Action on gelatin (increase in formaldehyde titration in cc.)	Action on collagen (mg. catgut dissolved)
Excreta preparation A:				
1	Unchanged	18	0·95	20
2	Kept 24 hours at 37°	18	0·9	12·5
3	Treated with kaolin	24	0·55	8
4	„ charcoal	24	0·5	4·5
Excreta preparation B:				
5	Unchanged	18	1·45	22
6	Kept 48 hours at 37°	18	1·05	11·5
7	„ 96 „	18	0·80	5·0
8	Treated with kaolin	18	0·95	9·5
9	„ charcoal	18	0·95	5·0

and, although no absolute separation has been effected, comparison of the results shows that differential adsorption has occurred. In Exps. 1, 8 and 9 the digestion of the gelatin has proceeded to the same extent but the amounts of catgut passing into solution are very different (20, 9·5 and 5·0 mg.). The digestion of collagen by the excreta seems therefore to be due to a separate enzyme which will be referred to as collagenase. Both charcoal and kaolin adsorb the collagenase more completely than the protease, charcoal being the more selective. The collagenase also appears to be less stable than the protease in alkaline solution at 37°. The results obtained by adsorbing the excreta of *Lucilia* larvae with kaolin and charcoal are in fair agreement with those of Ssadikov [1927] for pancreatin samples containing collagenase. However, Ssadikov found that kaolin destroyed the collagenase in pancreatin and obtained different results with glycerol extracts of the pancreas.

The origin of the collagenase.

The present author [1931] has already shown that the protease found in the excreta of *Lucilia* larvae is secreted by the mid-gut, but it is possible that the collagenase may be secreted elsewhere or produced by intestinal bacteria. To investigate this question, larvae were starved until the gut was empty, and the salivary glands, mid-guts and hind-guts from 15 larvae were dissected out, each lot being ground up to an emulsion with one drop of glycerol, diluted with 1 cc. of buffer at p_H 8·4 and tested with a small piece of catgut as substrate. Only the mid-gut preparation digested the collagen, and this showed very weak activity compared with the excreta. Furthermore, a preparation of the gut-contents of feeding larvae was found to digest gelatin actively, but not collagen. These results suggest that the collagenase may be produced by bacteria in the hind-gut, but this possibility was disproved by showing the presence of the enzyme in sterile larvae.

The technique of rearing *Lucilia* larvae aseptically will be described in

detail in a later publication; briefly the method used was to sterilise the eggs with 0·1 % mercuric chloride and rear the larvae on autoclaved brain. The residue from the growth of a batch of larvae shown to be sterile was tested by mixing it with an equal volume of buffer at p_H 8·4, adding thymol and catgut and incubating for 2 days at 37°. On examination the catgut showed definite evidence of digestion, being almost completely dissolved.

The collagenase therefore is produced by the larvae and not by bacteria, and there can be little doubt that it is secreted in the mid-gut. The inactivity of the gut-contents from fed larvae is probably due to adsorption of the enzyme by the food, as adding undigested food from the crop has been found to decrease the action of the excreta on collagen. The high ratio of collagenase to protease found in the excreta may be due to the disappearance of the protease, either by its being used up in digestion of the meal or by being partly readsorbed.

The influence of p_H *on the digestion of collagen by the excreta of* Lucilia *larvae.*

To investigate the effect of reaction on collagen digestion, equal lengths of uniform catgut were digested for 48 hours with the same volumes of an excreta preparation at different p_H values. Aliquots of the excreta were titrated with strong acid and alkali to the required reactions by means of a capillary pipette and mixed with equal volumes of buffer of the same p_H. Digestion was measured by estimating the loss of weight and p_H determined colorimetrically at the beginning and end of the experiment, the mean values being taken. The results are plotted in Fig. 1 and show that the optimum reaction for the

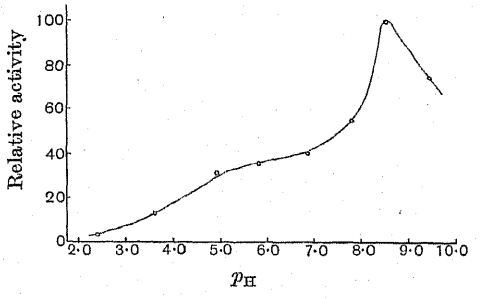


Fig. 1. p_H-activity curve of the digestion of collagen (catgut) by the excreta of *Lucilia* larvae.

collagenase lies between p_H 8·0 and 9·0. This reaction is more alkaline than the optimum found for the digestion of gelatin by the excreta, which was about p_H 7·5 [Hobson, 1931]. The collagenase is well adapted for working in blown meat, in which large amounts of ammonia develop, and the reaction generally lies between p_H 8·0 and 8·5.

Discussion.

The presence of a collagenase in the excreta may play an important part in the growth of *Lucilia* larvae on meat, by digesting the fine strands of connective tissue which surround each muscle fibre and represent to a large extent the structural elements of muscle tissue. The absence of a keratinase is hardly surprising, as this enzyme would only be advantageous in the case of myiasis on sheep, which is exceptional and possibly only a recently acquired habit. When developing in the wool of sheep, *Lucilia* larvae probably feed on the impurities present; if any digestion of keratin occurs, it must be the result of bacterial action.

An enzyme able to digest collagen has been described also by Shinoda [1928] from the gastric juice of the crayfish (*Astacus*). The enzymes from *Astacus* and from *Lucilia* larvae differ in their sensitivity to the reaction; with the gastric juice from *Astacus*, Shinoda found that digestion of collagen occurred over a wide range from p_H 4·0 to 8·0, the actual optimum at p_H 6·7 not being sharply defined; with the excreta from *Lucilia* larvae, the p_H-activity curve (Fig. 1) shows that digestion is limited to a narrow range on the alkaline side of neutrality, the optimum being well marked at about p_H 8·5. Shinoda measured the activity of the gastric juice of *Astacus* at different times during the secretion rhythm against various substrates, but failed to find evidence for the existence of separate proteolytic enzymes. He suggested that, until invertebrate proteases have been separated by modern methods of isolation, reference should be made to "enzyme actions" and not to "enzyme individuals." Shinoda partly based his argument on the fact that the protease from *Astacus*, by digesting collagen in neutral solution, combines to some extent the properties of pepsin and trypsin. However, this is not the case with the protease from *Lucilia* larvae, for adsorption with charcoal decreases the activity of the excreta towards gelatin and collagen to a different extent (Table II) and collagen digestion is, therefore, due to a separate enzyme. Furthermore, Wigglesworth [1928] has shown that the proteolytic enzymes of the cockroach are closely analogous to the corresponding vertebrate enzymes in many respects and was able to separate the tryptase and peptidase fractions by the method used by Waldschmidt-Leitz and Harteneck [1925] for the pancreatic enzymes. There is therefore good evidence that insect proteases include separate individuals which correspond closely to vertebrate trypsin and erepsin.

The reaction products of the collagenase from *Lucilia* larvae could not be determined, as complete separation of the collagenase and protease was not effected. Treatment with dilute acid in the cold or with water at 70° converts collagen into a hydrated form, hydrocollagen, which is digested by trypsin [Ssadikov, 1927], and this may be the end-product of the collagenase from *Lucilia* larvae. It may be noted that the ability of the excreta to digest collagen is not associated with increased action on other resistant proteins;

thus, elastin is digested more readily by trypsin than by the protease from the excreta (Table I).

SUMMARY.

1. The excreta of *Lucilia* larvae contain proteolytic enzymes which digest collagen and elastin but not keratin.

2. The enzyme which digests collagen is produced by the cells of the mid-gut and not by intestinal bacteria, since it occurs in sterile larvae.

3. The excreta digest collagen in alkaline solution, the optimum reaction being at about p_H 8·5. With increasing acidity the activity of the enzyme decreases and almost disappears at p_H 4·0.

4. The separate existence of a collagenase has been concluded from adsorption and stability experiments. The collagenase is less stable than the enzymes acting upon gelatin and is adsorbed to a greater extent by charcoal and kaolin.

I wish to express my appreciation to Dr V. B. Wigglesworth for suggesting this problem and for his active interest in the work. I am indebted to the Empire Marketing Board, for a grant which has financed this work entirely, and to Dr P. A. Buxton, for his interest in this research and hospitality in extending to me the facilities of this Department.

REFERENCES.

Hobson (1931). *J. Exp. Biol.* **8**, 109.
Sahli (1924). *J. Amer. Med. Assoc.* **82**, 51.
Shinoda (1928). *Z. vergl. Physiol.* **7**, 323.
Ssadikov (1927). *Biochem. Z.* **181**, 267.
Stankovic, Arnovlzevic and Matavulj (1929). *Z. physiol. Chem.* **181**, 291.
Waldschmidt-Leitz and Harteneck (1925). *Z. physiol. Chem.* **149**, 203.
Wigglesworth (1928). *Biochem. J.* **22**, 150.

CLVIII. THE OCCURRENCE OF VITAMIN D IN LAMPREYS (PETROMYZONTIDAE).

By ROBERT KENNETH CALLOW
and CATHERINE FÉLICITÉE FISCHMANN.

From the National Institute for Medical Research, London, N.W.3.

(*Received July 17th, 1931.*)

On the assumption that the storage of vitamin D in the livers of fishes is directly related to bone calcification, it might be expected that a decrease in the amount of vitamin D would be observed in passing from the Teleostei, or bony fishes, to the Elasmobranchii, or cartilaginous fishes, the skeleton of which contains very little calcium. The vitamin D storage should, on the same grounds, be still less in the Cyclostomata, a class of primitive vertebrates, distinct from fishes, in which the skeleton shows even smaller traces of calcification than that of elasmobranchs.

A number of species of Elasmobranchii has been investigated by Poulsson [1929, 1931] and by S. and S. Schmidt-Nielsen [1930], who established the presence of vitamin D in the livers, although in much smaller amount than in those of bony fishes, such as the cod. Poulsson suggested that the diffused ossification found in the vertebrae and the calcified deposits in the scales and teeth provided sufficient explanation of the presence of vitamin D. The vitamin D content of Cyclostomata has not previously been investigated by quantitative methods. Matzko [1929] examined one species, *Caspiomyzon wagneri*, which occurs in the Black Sea, in relation to its food value, and found that rats were protected from rickets by the large dose of 0·15 g. per day of the oil from the roasted flesh.

The principal subject of the present investigation has been the lampern, or river lamprey (*Petromyzon fluviatilis*), which is a fairly readily available species. In addition, a few specimens of the sea lamprey (*P. marinus*) have been examined. The liver oils, as well as those obtained from the ovary and from the eviscerated body of these two species, have been examined for vitamin D. The presence of vitamin A has also been investigated in view of the observations made by Mori [1904]. This author found that pills made of the lightly-cooked flesh of the sea lamprey were beneficial in the treatment of the infantile disease "hikan" (xerosis conjunctivae and keratomalacia) prevalent in Japan, although cod-liver oil was more efficacious. McCollum and Simmonds [1917] were the first to recognise this observation as an instance of the cure of vitamin A deficiency. The materials used by Mori have been referred to in the literature, through mistranslation, as "eel-fat" and "chicken-liver."

Experimental.

Material. The lamperns were caught in the River Severn at Tewkesbury in the months of March and April, 1930. At this season they ascend the river from the sea in order to spawn. Eighty-five specimens were received, mostly alive. Immediately after receipt the intestinal tract, liver, and gonads were removed. All the fish appeared to be sexually mature. The sea lampreys were caught in the same neighbourhood in May and June 1930, during the spawning migration. The season was a late and poor one. Only four specimens were received, all in good condition. We are indebted to Dr H. King of this laboratory for his assistance in obtaining these specimens through a local fisherman.

In order to compare elasmobranch fish with the Cyclostomata, some lesser spotted dogfish (*Scyllium canicula*) were examined. These were supplied by Plymouth Marine Biological Station in April and June, 1930. The females, received in April, were immature and the ovaries were rudimentary, but those received in June contained ova from 1 to 2 cm. in diameter.

Extraction of oil. Oils were obtained from the eviscerated bodies, livers, and ovaries of the lampreys, and from the livers of the dogfish. The same procedure was followed in all cases. The minced tissue was treated with 2 % aqueous potassium hydroxide solution (2 cc. to 1 g. of tissue) for 24 hrs. at room temperature, and then for 8–24 hrs. at 37°. After dilution with water, the solution was extracted with ether. Persistent emulsions were broken by the addition of a little alcohol. The extract was washed with water, dried over sodium sulphate, and the ether distilled off, finally under reduced pressure. The oils were stored in stoppered amber bottles in a refrigerator.

Determination of vitamin D. This was carried out by the method described by Bourdillon *et al.* [1931]. Rats rendered rachitic on Steenbock's rachitogenic diet were given comparable doses of the oils investigated and of a standard preparation of irradiated ergosterol, necessary dilutions being made in olive oil. X-ray photographs of the tibiae were taken at the beginning and end of the 14-day period of dosing and the degree of healing was determined by comparison with a calibrated radiographic scale. The antirachitic potencies are expressed in terms of the unit of the Medical Research Council, based on the standard solution issued by them.

Consideration of the "probable error" involved with the number of rats employed [*cf.* Bourdillon *et al.*, 1931] indicates that individual determinations may be subject to considerable error, but the figures obtained for a particular species, by summing the determinations on separate samples of the organs, are capable of indicating differences from another species with reasonable accuracy.

Determination of vitamin A. (*a*) *Biological measurements.* Owing to the small number of animals at our disposal and the limited quantity of most of the oils, only very rough and qualitative estimations of vitamin A contents

Table I. *Vitamin D determinations.*

Species ...	Lampern			Sea lamprey				Lesser spotted dogfish				
Tissue used	Eviscerated body	Liver	Ova	Eviscerated body	Liver (i)	(ii)	Ova	Liver (i) (April)	(ii) (April)	(iii) (June)	(iv) (June)	(v) (June)
Number (weight in g.) of fish used	♂ 11 (205) ♀ 13 (283)	30 (488) 19 (357)	38 (766)	1 (895) 1 (852)	2 (1505)	2 (1541)	2 (1541)	3 (—)	3 (—)	2 (1780)	4 (3180)	1 1 (1093)
Proportion of tissue to whole fish (%)	87	1·8	12·6	82	3·0	1·9	13·8			8·4	11	11·4
Yield of oil, weight in g.	9·9	0·31	10·6	53·7	10·7	1·2	26·4	104·4	155·3	68	103	62·2
Yield of oil, % of tissue	8·3	3·8	11	7·7	23·7	4·1	12·4	52	57	46	32	53·5
Antirachitic potency of oil, M.R.C. units/cc.	15	53	23·1	60	44	50	102	3·7	7	8·3	5	9
Antirachitic potency of tissue, M.R.C. units/g.	**1·2**	**2·0**	**2·5**	**4·0**	**10·2**	**2·05**	**9·9**	**1·9**	**4·0**	**3·8**	**1·6**	**4·8**
Number of pairs of rats used in each determination	4	3	7	3	2	3	3	5	3	3	3	3

Table II. *Vitamin A determinations.*

Species ...	Lampern						Sea lamprey				Lesser spotted dogfish				
Tissue used	Eviscerated body	Liver (i)	(ii)	(iii)	Ova	Sperm	Eviscerated body	Liver (i)	(ii)	Ova	Liver (i) (April)	(ii) (April)	(iii) (June)	(iv) (June)	(v) (June)
Number (weight in g.) of fish used	♂ 41 (693) ♀ 32 (640)	17 19 (741)	30 (488)	19 (357)	38 (766)	41 (693)	2 (1505) 2 (1541)	2 (1505)	2 (1541)	2 (1541)	3 (—)	3 (—)	2 (—)	4 (—)	1 1 (1093)
Proportion of tissue to whole fish (%)	86	1·9	1·5	2·7	12·6	7·6	80	3·0	1·9	13·8	—	—	8·4	11	11·4
Yield of oil, weight in g.	40·5	0·51	0·28	0·03	10·6	0·05	101·7	10·7	1·2	26·4	104·4	155·3	68	103	66·2
Yield of oil, % of tissue	8·4	3·5	3·9	0·3	11	0·1	6·0	23·7	4·1	12·4	52	57	46	32	53·5
Blue value of oil	0·3	12·3	15	16	6·4	3·4	0	Very low	2·8	0·4	0·11	0·05	0·2	0·15	0·4
Blue value of tissue per g.	0·63	10·8	14·2	1·2	17·6	0·085	0	—	2·8	1·24	1·43	0·71	2·3	1·2	5·4
Vitamin A potency: cod-liver oil = 100	8	—	—	—	6·6	—	—	—	—	—	20	—	—	—	11
Number of rats used	2	—	—	—	2	—	—	—	—	—	2	—	—	—	3

of a few oils were made. Growing rats were kept on a diet deficient in vitamin A until loss in weight took place. The oils to be tested were then administered in daily doses of 100 mm.^{3} and cod-liver oil, for comparison, was given in doses of 20 mm.^{3}, and the resumption of growth observed and measured over a period of 30 days. The relative potencies of the oils in the doses given were assumed to be proportional to the percentage increase in weight over this period, and the results are expressed as percentages of the potency of the cod-liver oil used, which had a blue value of 10·2.

(*b*) *Colorimetric measurements.* These are recorded, although we recognise the fact that a direct and quantitative relationship between the intensity of the antimony trichloride reaction and vitamin A content is not established. The blue values, referred to 40 mg. of oil, were determined with a Rosenheim-Schuster colorimeter by the method recommended by the sub-committee of the Pharmacopoeia Commission [1931], but determinations were sometimes made with undiluted oils.

The data of number and weight of fish, weight of tissues, and yield and potency of the oils obtained are given in detail in Tables I and II.

Discussion of results.

Comparison of the vitamin content of lampreys and other species.

The oils obtained from the body, liver, and ovary of both the lampern and the sea lamprey have been found to contain moderate amounts of vitamin D, and the antimony trichloride reaction, supplemented by a few biological tests, indicates that vitamin A is also generally present. The oil with the highest antirachitic potency was that from the ova of the sea lamprey, which was comparable with cod-liver oil. Only two specimens were examined, however, and the figures cannot be regarded as representative. The lesser spotted dogfish likewise yielded liver oils containing vitamins A and D, but in comparatively lower concentration. Since, however, different yields of oil are obtained from corresponding tissues of different species, it is preferable to base the comparison of species on the vitamin content calculated in terms of potency per g. of tissue; these figures are given in the eighth line of the Tables. The livers of lamperns, sea lampreys, and dogfish then appear to possess antirachitic potencies of roughly the same order. Even when account is taken of the relatively greater ratio of weight of liver to total body-weight in the dogfish, the vitamin D stored in its liver does not appear to be significantly greater than that stored in the livers of the lampreys. Moreover, lampreys contain, in addition, vitamin D in the body, which is rich in oil in comparison with that of the dogfish. The dogfish we examined yielded only 0·06 % of oil from the body, and sufficient was not obtained for determination of vitamin D.

Our results are, however, in agreement with those of previous workers who have shown that the livers of cartilaginous fish contain much less

vitamin D than those of bony fish such as the cod. The detailed and exact comparison of the vitamin content of different species is complicated by the influence of the factors of age, food, and sexual condition, as has been demonstrated in the case of the cod [Hess *et al.*, 1929; Drummond and Hilditch, 1930; Hawk, 1930], and it is our opinion that fuller attention must be paid to the variation due to these factors in each species, before definite conclusions can be drawn as to the variation of vitamin D content with species in relation to calcification, or other possible functions. The results we have obtained are based on a limited amount of material, and the evidence they yield must be used cautiously in considering the various problems involved in the calcium and fat metabolism of marine animals.

Relation of size of liver and oil content to sex. The proportion of weight of the liver to the total body weight in the female lampreys was about twice that in the male. The livers of the females were a translucent red, whilst those of the males were creamy and opaque. This difference in colour is probably due to difference in oil content, for the percentage yield of oil from female livers was about one-twelfth of that from male livers. The same difference in the yield of oil from the livers of the two sexes occurred in the sea lampreys. These constant differences in sexually mature lampreys of the same origin appear to be connected with the fat-storing function of the liver, and could perhaps be attributed to the greater transport of fat to the gonads which occurs in the female. Similar relations of size of liver and oil content to sex are known in the cases of the herring, salmon, and cod.

Distribution of vitamins and sex. Recently, investigations on cod have shown that the concentration of vitamin in the liver oil varies inversely with the amount of oil in the liver [Hess *et al.*, 1928, 1929; Drummond and Hilditch, 1930], from which the conclusion has been drawn that vitamins are not transferred to the gonads proportionately with the fat, but to a lesser degree. It might have been expected that comparison of sexually mature fish of different sexes would have shown some striking differences in the vitamin potencies of the liver oils, if it were further assumed that there had been a great difference in the transport of the fat to the gonads in the two sexes. Analysis of the data we have obtained shows, in fact, no evidence of considerable differences of this kind in the contents of either vitamin A or D.

Significance of the presence of vitamin D in lampreys. It is possible to argue that the presence of vitamin D in lampreys and elasmobranch fish is entirely fortuitous, and that it is absorbed from the foods with the fats and accompanies the latter in their distribution in the body. It seems much more attractive to suppose, however, that the vitamin is of physiological importance. Its function might then be explained by the presence of partly calcified structures. The cartilage of the head, for instance, is very hard, and we have been able to demonstrate qualitatively the presence of calcium in the horny teeth of *P. marinus*.

SUMMARY.

The oils extracted from the eviscerated body, liver and ovary of the lampern (*Petromyzon fluviatilis*) and the sea lamprey (*P. marinus*) have been found to contain vitamin D in moderate amount. Data for comparison with a typical cartilaginous fish have been obtained by examination of the liver oil from the lesser spotted dogfish (*Scyllium canicula*). The results indicate that there is little difference between the antirachitic potencies of the tissues of the species examined, although the antirachitic potencies of the livers are much lower than those of the cod. The bearing of the results on a possible correlation between vitamin D content and extent of calcification and on the transference of vitamin D in the body have been considered. Vitamin A is also found to be present in the oils examined.

We have great pleasure in recording our gratitude to Dr O. Rosenheim, who suggested this work, for his constant interest and advice.

REFERENCES.

Bourdillon, Bruce, Fischmann and Webster (1931). *Med. Res. Council Special Report Series*, No. 158.

Drummond and Hilditch (1930). Relative values of cod-liver oils from various sources. *Empire Marketing Board Publication*, No. 35, London.

Hawk (1930). *J. Biol. Chem.* **87**, *Sci. Proc.* xlviii.

Hess, Bills, Weinstock, Honeywell and Rivkin (1928). *Proc. Soc. Exp. Biol. Med.* **25**, 652.

— — and Honeywell (1929). *J. Amer. Med. Assoc.* **92**, 226.

McCollum and Simmonds (1917). *J. Biol. Chem.* **32**, 181.

Matzko (1929). *Biochem. Z.* **215**, 381.

Mori (1904). *Jahrb. Kinderheilk.* **59**, 175.

Pharmacopoeia Commission (1931). Report of Cod-liver Oil Colour Test Sub-Committee, London.

Poulsson (1929). *Strahlenther.* **34**, 648.

—— (1931). *Norsk Mag. Laegevidenskaben*, **92**, 125; *Bull. Hyg.* **6**, 438.

Schmidt-Nielsen and Schmidt-Nielsen (1930). *Z. physiol. Chem.* **189**, 229.

CLIX. THE OCCURRENCE OF SUGAR ALCOHOLS IN MARINE ALGAE. DULCITOL.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department, University College, London.

(*Received July 27th, 1931.*)

Stenhouse [1844] by isolating mannitol from a number of brown algae established for the first time the occurrence of a sugar alcohol in marine algae. In recent years the subject has been re-investigated by Kylin [1913, 1915], who attempted to estimate the amount of mannitol in different weeds by isolating the crystallised product and weighing it. In the course of an examination of the two brown seaweeds *Pelvetia canaliculata* and *P. canaliculata forma libera* [Haas and Hill, 1929] some difficulty was experienced in isolating the mannitol in crystalline form from the extracts obtained, a fact which was attributed to the presence of the non-crystallisable anhydride mannitan, which substance can also be classed as a sugar alcohol. The presence of both these substances was however established by the preparation of their respective benzylidene derivatives. Kylin [1915] in addition to examining a number of brown weeds also examined four red weeds for the presence of mannitol but in each case failed to find any of this substance. It therefore seemed to us to be of interest to ascertain whether the red alga *Bostrychia scorpioides* contained mannitol, since this weed grows on the salt marsh at Blakeney Point in close association with *P. canaliculata forma libera* and is thus exposed to conditions which are quite abnormal for a red weed. To this end the oven-dried weed was ground, sieved and extracted with boiling 60 % alcohol; the extract, after removal of the alcohol, was treated with basic lead acetate and filtered, and the filtrate was freed from lead by sulphuric acid. The resulting solution was then evaporated over a water-bath in the presence of calcium carbonate to ensure a neutral reaction. The residue thus obtained was a brown opaque pasty mass; it was extracted with boiling absolute alcohol, and the extract on evaporation gave a clear brown syrup, which, on standing for some weeks, deposited crystals. By the gradual addition of an equal volume of alcohol, in small quantities at a time with constant stirring, the crystals were separated from the brown supernatant fluid; they were found to be readily soluble in water but insoluble in alcohol and were accordingly dissolved in water, decolorised with charcoal and crystallised three times from alcohol and water, when they were obtained as glistening

monoclinic prisms melting at 186–187° (uncorr.). They gave the following result on analysis:

	C %	H %
Found	39·85	7·86
Calc. for $C_6H_{14}O_6$	39·56	7·69

When mixed with an authentic sample of dulcitol the melting-point remained unaltered but when mixed with a sample of mannitol of M.P. 163–165° the resulting mixture melted at 152–156°.

These facts in themselves are sufficient evidence that the substance was dulcitol but, to place the matter beyond doubt a small quantity was oxidised with nitric acid and was found to yield mucic acid M.P. 213°.

The occurrence of dulcitol has not previously been recorded in seaweeds, and this is indeed the first time that any sugar alcohol other than mannitol, or its anhydride, has been observed to occur in these plants. It is proposed to examine a number of other red weeds for this substance, but in the meantime it is worthy of note that the occurrence of dulcitol in a group of seaweeds which are known to contain galactose as a cell-wall constituent forms a striking parallel to the occurrence of mannitol in the brown weeds whose cell-wall has been shown to contain mannuronic acid [Bird and Haas, 1931].

REFERENCES.

Bird and Haas (1931). *Biochem. J.* **25**, 403.
Haas and Hill (1929). *Biochem. J.* **23**, 1005.
Kylin (1913). *Z. physiol. Chem.* **83**, 190.
—— (1915). *Z. physiol. Chem.* **94**, 337.
Stenhouse (1844). *Liebig's Ann.* **51**, 349.

CLX. A PRELIMINARY NOTE ON THE NITROGEN METABOLISM OF SEAWEEDS. GLUTAMIC ACID PEPTIDE.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department, University College, London.

(*Received July 27th, 1931.*)

In the course of an examination of the carbohydrate metabolism of certain brown seaweeds [Haas and Hill, 1929] it was found on testing concentrated extracts for sugars by means of Fehling's solution that the solution acquired a mauve tint. On investigation this was shown to be due to the presence of a substance in the extracts which gave a biuret reaction. The isolation of this substance has now been carried out on a somewhat larger scale than was at that time possible.

1 kg. of dried ground *Pelvetia canaliculata*, previously extracted with light petroleum, was covered with boiling water and kept warm over a small flame for about an hour; the weed was then strained through a fine wire sieve and treated a second time as before. The combined filtrates from the two extractions were then passed through calico and poured into an equal volume of absolute alcohol; the mucilaginous brown precipitate thus produced was strained through silk, and the liquid passing through was precipitated with basic lead acetate. After removing the precipitate the colourless solution was freed from lead by means of sulphuric acid, and the solution, while still acid with acetic acid, was treated with a slight excess of mercuric acetate and left to stand overnight. The precipitate A was then filtered, washed free from acid and decomposed with hydrogen sulphide; after removing the lead sulphide the solution was evaporated to small bulk under reduced pressure and finally dried in a vacuum. Thus prepared the product was a light yellow transparent syrup which gradually hardened to a resinous deliquescent solid (P). This substance was insoluble in organic solvents but was readily soluble in water to give an acid solution which on neutralisation however gave no precipitate with silver nitrate.

As the solid showed no tendency to crystallise after repeated attempts had been made to induce it to do so, it was decided to hydrolyse it, more especially as its general character suggested that it was a polypeptide.

Hydrolysis of the peptide (P).

To this end 1·8 g. of the substance was dissolved in 10 cc. of water and heated with 20 cc. of concentrated hydrochloric acid for 3 hours at 105°; the solution was evaporated to dryness over a water-bath and the residue taken up with water, filtered from some black solid and once more evaporated to dryness, when it weighed a little over 2 g. This material, dissolved in the least volume of concentrated hydrochloric acid, yielded 1·7 g. of crystalline solid which on analysis proved to be glutamic acid hydrochloride.

	C %	H %	N %	Cl %
Found	32·53	5·30	7·28	20·41
Calc. for $C_5H_9O_4N.HCl$	32·69	4·90	7·63	19·89

The mother-liquor from the glutamic acid hydrochloride weighing about 0·6 g. contained ammonium chloride; it was treated with freshly precipitated silver carbonate and, after filtering and saturating with hydrogen sulphide, evaporated to dryness in a vacuum. The resulting syrup on drying yielded a colourless resinous mass which was now free from chloride but still yielded ammonia in quantity on addition of dilute caustic soda in the cold and gave an immediate crystalline precipitate with platinic chloride, showing it to be an ammonium salt. It contained 12·72 % of nitrogen but owing to the limited amount of material available it could not be further identified.

Characterisation of the peptide (P).

That the substance was a peptide was shown by the fact that its amino-nitrogen content was increased eightfold after hydrolysis, from which it may be concluded to be an octapeptide.

Determinations of total and amide nitrogen gave the figures 15·07 % and 3·62 % respectively. In addition it was found that the substance had a strong acid reaction which was not increased by the addition of a neutral solution of formaldehyde. Heating over a boiling water-bath with excess of sodium hydroxide and titrating back the excess of alkali gave an equivalent of 209·2.

These figures coupled with the fact that after hydrolysis there remains, in addition to glutamic acid, a certain amount of material which has not as yet been identified make it unsafe to speculate further upon the probable constitution of the peptide, more especially as it has not been found possible as yet to effect any real purification, and the substance is known to contain a trace of ash. It is however hoped to subject this material to further study when more is available.

Occurrence of the peptide in other algae.

On extending the search for this peptide to other brown seaweeds it was found that most of those examined gave the biuret reaction but that the intensity of the reaction fell off with the depth of immersion of the weed, as

may be seen from the following table, in which the number of + signs indicates approximately the relative strength of the biuret reaction obtained.

Pelvetia canaliculata f. libera	+ +
Pelvetia canaliculata	+ +
Fucus vesiculosus	+
Fucus serratus	+
Ascophyllum nodosum	+
Halydris siliquosa	Weak
Himanthalia lorea	Absent
Laminaria spp.	Absent

Extracts prepared from the following green and red weeds all failed to give the biuret reaction: *Enteromorpha intestinalis* (both fresh water and sea water), *Ulva lactuca*, *Chaetomorpha* sp. (fresh water), *Bostrychia scorpioides*, *Chondrus crispus*, *Polysiphonia fastigiata* and *Rhodymenia palmata*.

Physiological significance of the peptide.

In attempting to account for the physiological significance of this substance it is worthy of note that polypeptides, with the possible single exception of glutathione, are generally regarded as transition products which only occur occasionally as intermediate products under conditions of somewhat abnormal metabolism, whereas the evidence so far obtained indicates that it is a stable substance of normal occurrence.

It may be recalled that asparagine and glutamine, which also give the biuret reaction, have been found in seedlings belonging to a large number of widely diverging families, but in such plants these substances appear to owe their presence to conditions of carbohydrate starvation, due to insufficient illumination or other conditions tending to reduce the supply of carbohydrate. Whether the conditions of intermittent desiccation to which the brown seaweeds are exposed and the consequent interruption in photosynthesis are sufficient to account for the appearance of this substance it is not as yet possible to say, though the diminution of the intensity of the reaction with increasing normal depth of immersion of the weeds appears to support this view. It is intended to investigate this matter.

Occurrence of trimethylamine.

The filtrate from the precipitate A (p. 1472), after being freed from mercury with hydrogen sulphide and from sulphuric acid by means of baryta, was evaporated under reduced pressure. The resulting syrup was rendered alkaline with sodium hydroxide and steam-distilled; the distillate was collected in hydrochloric acid and then evaporated to dryness. The residue after drying in a desiccator was extracted with chloroform, and the extract on evaporation left a small quantity of material which gave a crystalline precipitate with a solution of iodine in potassium iodide in the presence of hydrochloric acid; in addition, it gave a brown crystalline precipitate with gold chloride solution containing sodium bromide, tests which, coupled with the characteristic smell

given off on the addition of sodium hydroxide, establish the presence of trimethylamine.

SUMMARY.

1. A preliminary survey of the seaweeds growing round the coast of Britain has shown that the brown weeds exhibit certain peculiarities in their nitrogen metabolism which distinguish them from the green and red weeds growing above and below them.

2. Aqueous extracts of certain brown seaweeds, after appropriate treatment, give a marked biuret reaction, the intensity of which diminishes with the depth of immersion at which the weeds grow.

3. The material responsible for this reaction has been isolated and proves to be an octapeptide of glutamic acid.

4. No similar product has been found in the red or green weeds.

The authors desire to express their indebtedness to Miss M. Parke for supplying them with material.

REFERENCE.

Haas and Hill (1929). *Biochem. J.* **23**, 1000.

CLXI. THE BIOLOGICAL VALUES OF THE PROTEINS OF BREADS BAKED FROM RYE AND WHEAT FLOURS ALONE OR COMBINED WITH YEAST OR SOYA BEAN FLOUR.

By STANISŁAW KAZIMIERZ KON and ZOFJA MARKUZE.

From the Biochemical Department, State School of Hygiene, Warsaw.

(*Received August 3rd, 1931.*)

In the course of a study of the nutritional values of various Polish foodstuffs, carried out by us in 1929 and 1930 on behalf of the Flour and Bread Committee of the Polish Ministry of Interior, the biological values of the proteins (N × 6·25) of various breads commonly consumed in Poland and of several experimental ones have been ascertained. It is thought worth while to present here such of the results as may be of more general interest.

The biological analysis of 17 products is discussed in the present paper. These may be divided into three groups, (1) various breads commonly consumed in Poland; (2) experimental breads; (3) soya bean flour and baker's yeast.

Breads commonly used were purchased from the Municipal Bakeries in Warsaw. Experimental breads were baked in the Department of Technology of Fermentation and Foodstuffs, Warsaw Technical Institute, under the supervision of Prof. W. Iwanowski who co-operated with us.

In the preparation of the experimental breads we have been interested in combinations of rye and wheat flours with varying amounts of baker's yeast and of soya bean flour. The two latter substances have recently attracted considerable attention as highly concentrated sources of cheap vegetable protein.

The baker's yeast used as admixture was a brand well known in Poland. The soya bean flour "Edelsoja" was purchased from the Oesterreichische Soja-Aktiengesellschaft in Vienna. It is manufactured under a secret process (Berczeller) and is stated by the manufacturers to keep indefinitely without becoming rancid although it contains about 20 % of fat.

The nutritional value of this product and especially its vitamin and mineral contents have been discussed by one of us in a separate publication [Kon, 1930]. As we have made no direct comparisons we do not know whether the biological value of the proteins of the soya bean flour used in the present study is identical with or different from that of the proteins originally present in the soya bean.

Experimental.

The numerical method proposed by Osborne, Mendel and Ferry [1919] was used for the estimation of the biological value of the proteins of the various products studied. The rats were kept singly in cylindrical cages on screens and

Table I.

No.	Product	Protein content* (N × 6·25) %	Remarks
1	White wheat bread	11·0	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 140 cc. of 2½ % solution of NaCl and 5 g. fresh baker's yeast to the loaf
2	Wheat rolls	11·5	From the Municipal Bakeries
3	White wheat-yeast bread I	14·1	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 140 cc. of a 2½ % solution of NaCl and 90 g. of fresh baker's yeast to the loaf
4	White wheat-yeast bread II	15·8	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 150 cc. of a 2½ % solution of NaCl and 140 g. of fresh baker's yeast to the loaf
5	Soya flour-wheat bread I	14·7	Baked by Prof. Iwanowski. 280 g. of a mixture of white wheat flour (90 %) and soya bean flour† (10 %), 150 cc. of a 2½ % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
6	Soya flour-wheat bread II	18·5	Baked by Prof. Iwanowski. 280 g. of a mixture of white wheat flour (80 %) and soya bean flour† (20 %), 160 cc. of a 2½ % solution of NaCl and 5 g. fresh baker's yeast to the loaf
7	Rye bread from 70 % rye flour	6·6	From the Municipal Bakeries. Prepared by acid fermentation. (No yeast added, fermentation started by the addition of fermented dough from previous baking.)
8	Crust of bread No. 7	6·6	
9	Crumb of bread No. 7	7·0	
10	Rye bread from 70 % rye flour	6·3	Baked by Prof. Iwanowski. Same method as for bread No. 7
11	Rye-yeast bread from 70 % rye flour	8·6	Baked by Prof. Iwanowski. 280 g. 70 % rye flour, 140 cc. of a 3 % solution of NaCl and 50 g. of fresh baker's yeast to the loaf
12	Soya flour-rye bread I	10·6	Baked by Prof. Iwanowski. 280 g. of a mixture of 70 % rye flour (90 %) and soya bean flour† (10 %), 160 cc. of a 3 % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
13	Soya flour-rye bread II	14·8	Baked by Prof. Iwanowski. 280 g. of a mixture of 70 % rye flour (80 %) and soya bean flour† (20 %), 170 cc. of a 3 % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
14	Military bread (rye)	9·1	From the Municipal Bakeries. 82 % rye flour. No yeast. (Same method as for bread No. 7)
15	Country dark rye bread (whole-meal)	9·9	Representative sample of country bread baked from home-milled rye flour (approx. 100 %), prepared by acid fermentation
16	Soya bean flour	45·5	A commercial preparation containing about 20 % fat, marketed under the name of "Edelsoja" by the Soja-Aktiengesellschaft in Vienna
17	Baker's yeast	45·6	Same brand as used for admixture to all the yeast breads described above

* On the dry basis. † As described under 16.

the diets were offered in glass feeders with metal hoods to prevent scattering. All diets containing raw starch were mixed with water and heated on the water-bath until dextrinised. Diets containing only bread and the salt mixture were mixed with water without cooking. Tap-water was offered *ad lib.* in glass bulbs. Each rat was given daily, separately from the diet, 3 drops of cod-liver oil and 0·15–0·2 cc. of an Osborne and Wakeman [1919] watery extract from brewer's yeast equivalent to 0·3–0·4 g. of dry yeast and containing 4·8–6·4 mg. N. The extract was omitted in the case of diets containing an abundance of vitamin B complex.

Potato starch was used in the compounding of the rations as it can be obtained in a state of greater purity (lower nitrogen content) than other commercial starches.

Every day the food residues were removed from the food cups, combined with any scattered food, collected on filter-paper on the trays and dried to constant weight. On Saturdays double rations were issued, no cleaning being done on Sundays. The experiments were, on the average, of 30 days' duration.

In Table I a list of the various products analysed and their nitrogen contents are given. Table II presents the composition of the various diets used in

Table II. *Composition of diets.*

All percentages on dry basis.

No.	Diet		%	%	%	%
1	White wheat bread	Protein (N × 6·25) level	8·6	10·4	—	—
		Bread	76·2	95·0	—	—
		Butter fat	10·0	—	—	—
		Salts*	5·0	5·0	—	—
		Potato starch	8·8	—	—	—
2	Wheat rolls	Protein (N × 6·25) level	6·4	8·2	10·9	—
		Rolls	53·0	70·5	95·0	—
		Butter fat	10·0	10·0	—	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	32·0	14·5	—	—
3	Wheat-yeast bread I	Protein (N × 6·25) level	9·1	10·4	12·9	—
		Bread	61·5	71·0	95·0	—
		Butter fat	10·0	10·0	—	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	23·5	14·0	—	—
4	Wheat-yeast bread II	Protein (N × 6·25) level	8·3	9·6	12·3	—
		Bread	51·2	63·3	76·0	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	33·8	21·7	9·0	—
5	Soya flour-wheat bread I	Protein (N × 6·25) level	8·1	10·2	11·9	—
		Bread	54·4	68·0	81·6	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	30·6	17·0	3·4	—
6	Soya flour-wheat bread II	Protein (N × 6·25) level	8·5	10·1	15·3	—
		Bread	43·2	54·0	81·0	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	41·8	31·0	4·0	—

* Steenbock's salt mixture No. 40.

Table II (*contd.*).

No.	Diet		%	%	%	%
7	70 % rye bread	Protein (N × 6·25) level	6·4	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
8	Crust of bread No. 7	Protein (N × 6·25) level	6·3	—	—	—
		Crust	95·0	—	—	—
		Salts	5·0	—	—	—
9	Crumb of bread No. 7	Protein (N × 6·25) level	6·7	—	—	—
		Crumb	95·0	—	—	—
		Salts*	5·0	—	—	—
10	70 % rye bread	Protein (N × 6·25) level	6·0	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
11	Rye-yeast bread	Protein (N × 6·25) level	8·2	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
12	Soya flour-rye bread I	Protein (N × 6·25) level	8·0	10·2	—	—
		Bread	75·5	95·0	—	—
		Butter fat	10·0	—	—	—
		Salts*	5·0	5·0	—	
		Potato starch	9·5	—	—	—
13	Soya flour-rye bread II	Protein (N × 6·25) level	8·1	10·1	11·9	—
		Bread	54·0	67·6	81·1	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	31·0	17·4	3·9	—
14	Military bread	Protein (N × 6·25) level	8·6	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
15	Country whole-meal rye bread	Protein (N × 6·25) level	9·7	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
16	Soya bean flour	Protein (N × 6·25) level	8·5	10·6	12·3	15·3
		Soya flour	17·6	22·0	26·4	33·0
		Butter fat	10·0	10·0	10·0	10·0
		Salts*	5·0	5·0	5·0	5·0
		Rice starch	67·4	63·0	58·6	52·0
17	Baker's yeast	Protein (N × 6·25) level	8·4	10·0	12·1	—
		Yeast	17·5	21·9	26·3	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Rice starch	67·5	63·1	58·7	—

* Steenbock's salt mixture No. 40.

the course of the study, while in Table III the biological values of the proteins are summarised.

Discussion.

Owing to the low nitrogen content several breads have been fed to rats only at one level of protein intake. This level has not necessarily been constant for all the breads studied in the present work. A direct comparison of the biological values of the nitrogen contained in these breads is therefore not possible. While our figures indicate that rye breads in general are superior, as far as the biological value of the protein goes, to wheat products, and that darker rye breads made from less refined flour are in turn superior to lighter varieties, we do not feel entitled, for reasons stated above, to draw definite

Table III.

No.	Diet	Protein (N × 6·25) content %	Sex of rats	Days	Total intake, g. Food*	Total intake, g. Protein*	Initial weight g.	Final weight g.	Gain in weight g.	Gain per g. protein	Average gain per g. protein
1	White wheat bread	8·6	♀	29	229·1	19·7	65	77	12	0·61	0·81
			♂	31	325·5	28·0	85	109	24	0·86	
			♂	30	278·6	24·0	71	94	23	0·96	
		10·4	♀	31	274·7	28·6	70	96	26	0·91	1·09
			♂	31	285·3	29·7	72	103	31	1·04	
			♀	31	274·2	28·5	69	107	38	1·33	
2	Wheat rolls	6·4	♀	31	283·5	18·1	50	68	18	0·99	0·98
			♀	31	307·5	19·7	57	76	19	0·96	
		8·2	♂	31	315·0	25·8	57	86	29	1·12	1·06
			♀	31	318·8	26·1	54	80	26	1·00	
		10·9	♀	31	434·7	47·4	51	103	52	1·10	1·13
			♂	31	409·9	44·7	55	107	52	1·16	
3	White wheat-yeast bread I	9·1	♀	29	261·5	23·8	55	93	38	1·60	1·65
			♂	28	173·7	15·9	42	69	27	1·70	
		10·4	♀	29	327·0	34·0	75	138	63	1·85	1·86
			♀	29	338·9	35·2	74	140	66	1·87	
		12·9	♀	31	288·0	37·3	52	119	67	1·80	1·90
			♂	31	381·0	49·3	54	152	98	1·99	
4	White wheat-yeast bread II	8·3	♀	31	219·7	18·3	52	93	41	2·24	1·87
			♂	31	246·4	20·5	61	92	31	1·51	
		9·6	♀	31	211·6	20·2	54	89	35	1·73	1·60
			♂	31	205·2	19·7	53	82	29	1·47	
		12·3	♀	30	219·6	27·0	48	94	46	1·70	1·91
			♀	31	442·6	54·4	54	170	116	2·13	
5	Soya flour-wheat bread I	8·1	♀	30	248·5	20·1	57	103	46	2·28	1·97
			♀	33	294·8	23·9	71	111	40	1·67	
		10·2	♂	30	238·7	24·4	60	100	40	1·64	1·62
			♀	30	244·4	25·0	65	105	40	1·60	
		11·9	♀	30	229·0	27·3	57	96	39	1·43	1·46
			♂	30	218·7	26·0	57	96	39	1·49	
6	Soya flour-wheat bread II	8·5	♀	30	231·1	19·6	75	102	27	1·37	1·33
			♀	30	264·5	22·5	80	109	29	1·29	
		10·1	♀	30	300·5	30·4	85	138	53	1·74	1·79
			♀	30	322·9	32·6	75	135	60	1·84	
		15·3	♀	30	292·2	44·7	61	152	91	2·03	1·95
			♂	30	265·2	40·6	61	137	76	1·87	
7	70 % rye bread	6·4	♀	31	219·0	14·0	47	63	16	1·14	1·25
			♀	31	227·4	14·6	41	61	20	1·37	
			♀	31	202·4	12·9	45	61	16	1·24	
8	Crust of bread No. 7	6·3	♂	30	236·0	14·9	57	77	20	1·34	0·83
			♂	31	205·5	13·0	65	73	8	0·62	
			♂	31	213·5	13·5	65	72	7	0·52	
9	Crumb of bread No. 7	6·7	♂	31	303·4	20·3	66	95	29	1·42	1·26
			♂	31	278·6	18·7	67	89	22	1·17	
			♂	30	239·3	16·0	64	83	19	1·18	
10	70 % rye bread	6·0	♂	30	272·0	16·3	60	80	20	1·23	1·11
			♀	30	268·0	16·1	56	73	17	1·06	
			♀	30	257·7	15·5	56	72	16	1·03	
11	Rye yeast bread	8·2	♀	31	205·0	16·8	54	67	13	0·77	1·07
			♂	31	330·3	27·1	48	98	50	1·84	
			♂	30	188·5	15·4	59	68	9	0·58	
12	Soya flour-rye bread I	8·0	♀	31	308·9	24·7	55	110	55	2·22	2·04
			♀	31	185·0	14·8	40	62	22	1·50	
			♂	31	203·3	16·3	43	82	39	2·39	
		10·2	♀	31	207·4	21·2	43	75	32	1·51	1·59
			♂	31	314·3	32·1	51	105	54	1·68	

* Water-free basis.

Table III (*contd.*).

No.	Diet	Protein (N × 6·25) content %	Sex of rats	Days	Total intake, g. Food*	Total intake, g. Protein*	Initial weight g.	Final weight g.	Gain in weight g.	Gain per g. protein	Average gain per g. protein
13	Soya flour-rye bread II	8·1	♀	31	286·2	23·2	59	116	57	2·46	2·43
			♀	31	273·0	22·1	55	108	53	2·40	
		10·1	♀	30	231·9	23·4	53	101	48	2·05	1·95
			♀	30	208·4	21·1	48	87	39	1·85	
		11·9	♀	30	237·7	28·3	55	108	53	1·87	2·02
			♀	30	269·7	32·1	53	123	70	2·18	
14	Military bread (rye)	8·6	♀	31	217·2	18·7	44	72	28	1·50	1·75
			♂	31	331·7	28·5	50	103	53	1·85	
			♂	31	431·2	37·1	61	132	71	1·91	
15	Whole-meal country rye bread	9·7	♀	31	268·3	26·0	47	80	33	1·27	1·29
			♂	31	230·8	22·4	42	67	25	1·11	
			♂	31	331·0	32·1	57	105	48	1·49	
16	Soya bean flour	8·5	♀	30	261·8	22·3	61	95	34	1·53	1·64
			♀	30	370·1	31·5	65	120	55	1·75	
		10·6	♂	30	319·2	33·7	74	126	52	1·55	1·57
			♀	30	299·3	31·6	68	118	50	1·59	
		12·3	♂	30	300·8	37·0	72	133	61	1·65	1·51
			♀	29	247·1	30·4	70	112	42	1·38	
		15·3	♂	30	306·1	46·8	53	135	82	1·75	1·58
			♀	30	312·4	47·8	49	119	70	1·46	
			♂	30	316·8	48·5	52	129	77	1·52	
17	Baker's yeast	8·4	♀	30	326·0	27·4	60	100	40	1·46	1·48
			♀	29	273·7	23·0	63	97	34	1·49	
		10·0	♀	30	257·0	25·7	61	99	38	1·48	1·32
			♂	29	287·0	28·7	72	105	33	1·15	
		12·1	♀	30	268·1	32·4	67	107	40	1·23	1·36
			♂	29	300·5	36·4	72	126	54	1·49	

* Water-free basis.

conclusions from the results of these tests. Our data would also seem to indicate that whole-meal rye bread baked from 100 % rye flour is inferior with regard to the biological value of its proteins to dark rye bread prepared from 82 % flour. This might be due to the presence in the former bread of rather large amounts of indigestible roughage derived from the grain husks and interfering with the normal utilisation of the proteins.

While we are thus inclined to present only a series of figures for some of the breads studied without subjecting the former to comparison or discussion, we feel that a more detailed review will be permissible in the case of other breads where biological values obtained at several levels of protein intake are available.

Two wheat-yeast breads, one containing approximately 8 %, the other approximately 12 % of yeast (on the dry basis) have been prepared and tested at three levels of protein intake. Yeast alone was fed at three similar levels, while the two lower levels were used for the study of the biological value of the nitrogen of wheat bread alone. The results are presented in Table III, Nos. 1, 3, 4 and 17. It is evident that in both cases and at all levels of protein intake the combinations of wheat flour and baker's yeast yielded higher biological values than either of the components. It may therefore be concluded that a supplementary relation exists between the proteins of white wheat flour and

those of baker's yeast, when mixed in the approximate proportion of 7 to 1 and 11 to 1.

Owing to technical difficulties in the preparation of rye-yeast breads, only one such bread could be prepared, containing a rather low percentage of yeast (about 4·5 %). When fed at the only level of 8·2 % of protein this bread caused a gain in weight of 1·07 g. per g. of protein ingested, lower than that caused by yeast alone at a corresponding level (8·4 %) and of about the same order as given by rye bread at a 6·0 % level (Table III, Nos. 10, 11 and 17). The results for the rye-yeast bread were very irregular, one rat gaining at a rate of 1·84 g. per g. of protein ingested while two other rats made gains of only 0·77 and 0·58. It is obviously impossible to judge from these results whether or not there exists a supplementary relation between the proteins of rye and those of baker's yeast.

Two soya bean flour-wheat flour breads have been prepared, the ratios of the constituents being 1 to 9 and 1 to 4 respectively. Each of these breads has been fed to rats at three levels of protein intake. Soya bean flour alone has been fed at four levels. As already stated the basal wheat bread was fed at two levels of protein intake (Table III, Nos. 1, 5, 6 and 16). For both soya-wheat breads and at all levels studied the results are better than for wheat bread alone. For the soya bean flour-wheat flour bread I (ratio of constituents 1 to 9) a maximum gain of almost 2 g. per g. of protein ingested has been found at an approximately 8 % level of intake, decidedly superior to the value found at this level for soya bean flour alone and more than double the gain obtained for the basal wheat bread at a similar protein concentration. The second soya-wheat bread (ratio of constituents 1 to 4) produced maximal gains at the highest level of protein intake definitely surpassing those found for soya flour alone at the same level. The basal wheat bread was not fed at this level but it seems exceedingly unlikely that at any plane of protein intake it could produce gains surpassing or equalling the maximum shown by the combination. These results indicate a definite supplementary relation between the proteins of white wheat flour and of soya bean flour when mixed in the approximate proportions of 9 to 1 and 4 to 1.

Two soya bean flour-rye flour breads have been subjected to biological analysis (Table III, Nos. 12 and 13). The ratio of the constituents was the same as in the case of the soya-wheat breads. A basal rye bread was prepared from the same batch of rye flour as used for the combined breads (Table III, No. 10). Owing to the low nitrogen content this bread was fed at only one level of protein intake, soya-rye bread I (ratio of constituents 1 to 9) was tested at two levels while the second combined bread (ratio 1 to 4) was fed at three levels. Maximal values were found for both experimental breads at an 8 % level of protein intake. These values are definitely superior to those found for the proteins of soya bean flour at a similar level. In fact the second experimental bread gave at all three levels of protein intake values superior to those found at corresponding levels for the soya bean nitrogen. As rye bread

alone was not fed at these levels, no direct proof of a supplementary relation between the proteins of the rye flour and of soya bean flour is available. There seems, however, to be little doubt that such a relationship exists, as both the values found for military bread baked from 82 % rye flour and fed at an 8·6 % level and for whole-meal bread (100 % flour) fed at a 9·7 % level and reported in the present work, as well as values found by Osborne and Mendel [1920] for the proteins of the whole rye kernel at 8 and 10 % levels, are definitely lower than the values reported here at corresponding levels. In fact the gain of 2·4 g. per g. of protein ingested produced by the soya-rye combination at an 8 % level of intake is exceptionally good for vegetable proteins.

It will be noted that in the preparation of the experimental breads containing soya bean flour a small amount of foreign nitrogen was necessarily introduced through the addition of yeast (less than 0·5 % of the dry matter) in the process of baking. A supplementary action of this nitrogen cannot be ruled out. We doubt however whether it could have affected the results to any measurable extent at such a low concentration.

In order to verify whether the high temperature to which the crust of bread is exposed in the process of baking has any deterimental influence upon the biological value of the proteins, crumb and crust have been studied separately. Loaves of rye bread obtained from the Municipal Bakeries were split in halves, and the crust separated from the crumb as carefully as possible, first by hand, and then by cautiously cutting away all the caramelised brown layer of crust. Crust, crumb, and the whole bread were studied at the same plane of protein intake (Table III, Nos. 7, 8 and 9) and the biological value of the nitrogen of the crust has been found to be lower than that of the crumb or of the whole bread. There is no difference between whole bread and crumb, showing that probably the injury caused by heat in the proteins of the crust is compensated when they are combined with the proteins of the crumb. In a comprehensive study of the effect of heat on the biological value of proteins Morgan [1931] has recently reported a similar decrease of the biological value of the nitrogen of the crust as compared with the crumb.

It is interesting to note that in the case of soya bean flour a change of the level of protein intake from 8·5 % to 15·3 % caused hardly any difference in the biological value of the protein, making it impossible to select the level giving the maximal gain in weight per g. of protein ingested. The differences are also very slight for wheat rolls (three levels of intake from 6·4 % to 10·9 %) and for baker's yeast (three levels of intake from 8·4 % to 12·1 %). This is in harmony with similar findings of Morgan [1931] in the case of wheat gluten.

SUMMARY.

1. The biological values of the proteins (N × 6·25) of various natural and experimental breads, of baker's yeast and of soya bean flour have been determined by the numerical method of Osborne, Mendel and Ferry.

2. A supplementary relation exists between the proteins of white wheat flour and those of baker's yeast.

3. A supplementary relation also exists between the proteins of white wheat flour and soya bean flour.

4. There is a strong indication that a supplementary relation exists between the proteins of rye flour and of soya bean flour.

5. The biological value of the nitrogen of crust was found to be lower than that of crumb or of whole bread.

REFERENCES.

Kon (1930). Mąka i Pieczywo. Sprawozdanie z działalności Głównej Komisji do badania mąki i pieczywa. Ministerstwo Spraw Wewnętrznych. Warszawa. Pp. 101–135.

Morgan (1931). *J. Biol. Chem.* **90**, 771.

Osborne and Mendel (1920). *J. Biol. Chem.* **41**, 275.

—— Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.

—— and Wakeman (1919). *J. Biol. Chem.* **40**, 383.

CLXII. THE HEAT-INACTIVATION OF THE PROTEINASE OF THE PANCREAS.

By JAMES PACE.

From the Muspratt Laboratory of Physical and Electro-chemistry, University of Liverpool.

(*Received August 5th, 1931.*)

Introduction.

While previous work of the writer, upon the heat inactivation of trypsin, was in progress, it was shown by Waldschmidt-Leitz and Purr [1929] that trypsin itself is not an individual enzyme, but that it may be resolved into two components—a proteinase and a carboxypolypeptidase.

It was found by these authors that the proteinase, although completely inactive alone, is capable, when associated with enterokinase, of hydrolysing proteins and certain of their degradation products. The carboxypolypeptidase, in the absence of enterokinase, attacks a number of polypeptides and some dipeptides, which possess a free carboxyl group in the molecule, but will not attack a protein. Enterokinase increases the activity of carboxypolypeptidase. This work of Waldschmidt-Leitz and Purr has a distinct bearing upon the interrelation of trypsin and enterokinase. It is of interest to recall, very briefly, the views which have been held about this problem.

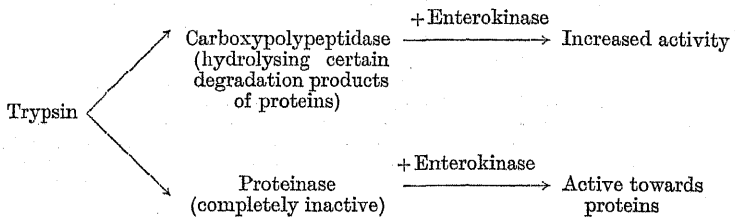
(*a*) *The "classical" view.* It was considered that an inactive zymogen, trypsinogen is produced in the pancreas. The trypsinogen is enzymically transformed into trypsin by enterokinase, and the trypsin thus produced is able to hydrolyse proteins. This may be expressed thus:

$$\text{Trypsinogen (inactive)} \xrightarrow[\text{as an enzyme}]{\text{Enterokinase acting}} \text{Trypsin (active)}$$

(*b*) *The view of the Willstätter school up to 1930.* According to this view the pancreas produces an enzyme, trypsin, which, although able to hydrolyse certain of the products of protein degradation, is inactive towards proteins themselves. Trypsin forms an addition compound with enterokinase, namely, trypsin-kinase, which readily hydrolyses proteins:

$$\text{Trypsin (inactive towards proteins, but hydrolysing certain degradation products)} + \text{Enterokinase} \longrightarrow \text{Trypsin-kinase (active towards proteins)}$$

(*c*) *The view arising from the work of Waldschmidt-Leitz and Purr.* This may be summarised thus:



Thus proteinase is analogous to the classical trypsinogen.

In the earlier work of the writer [1930] the investigation of the heat-inactivation of trypsin was carried out with solutions containing 24 % glycerol, this circumstance arising from the technique used in preparing the trypsin solutions. Further experiments were planned to study the effect of varying the glycerol concentration so that, by extrapolation, a value of the critical increment of the process in solutions containing no glycerol could be obtained, since such a value would obviously be of importance from the point of view of the behaviour of purely aqueous systems. From the work of Waldschmidt-Leitz and Purr it was apparent, however, that it would be more satisfactory to examine not "trypsin" itself but one of its components, namely proteinase, this component being chosen because it is more closely analogous to the classical trypsinogen (the carboxypolypeptidase component being more analogous to erepsin).

In the method evolved by Waldschmidt-Leitz and Purr for the isolation of the proteinase, we are left, after numerous adsorptions, with this enzyme in solution. Clearly any adjustment of the glycerol concentration would involve still further dilution, with a resulting diminution in concentration of enzyme. In view of this it was evident that as an important preliminary step an attempt should be made to carry the Waldschmidt-Leitz and Purr treatment further by adsorbing proteinase on a suitable adsorbent, say kaolin. The proteinase could then be eluted from the kaolin, always by the same volume of liquid, but the percentage of glycerol in that volume could be previously adjusted. In this way for example the enzyme could be eluted from the kaolin by an aqueous solution, containing no glycerol, and thus the critical increment of the heat inactivation process in water could be determined directly instead of by extrapolation.

Experimental.

(*a*) *The preparation of alumina* C_γ. The alumina used in the isolation of the proteinase was a sample of alumina C prepared according to the technique of Willstätter and Kraut [1923] about 12 months previously and since then kept under distilled water. It was therefore completely transformed into the C_γ modification.

(*b*) *Isolation of the proteinase.* This was carried out in principle according

to the method of Waldschmidt-Leitz and Purr, the actual procedure adopted being as follows. 100 cc. of ice-cold glycerol extract of dried pancreas were brought to p_H 4·0 by addition of 75 cc. of ice-cold buffer solution. (The buffer solution was made up by mixing 17·6 cc. *N* acetic acid and 2·04 cc. *N* sodium acetate and diluting to 100 cc.). 5 cc. of alumina C_γ suspension (containing 105 mg. Al_2O_3) were then added to the buffered enzyme extract. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. The clear supernatant liquid was then treated in exactly the same way with a second 5 cc. of alumina C_γ suspension. This operation, which was repeated four times in all, removes the dipeptidase and aminopolypeptidase. The clear solution from the final adsorption was then brought to p_H 7·0 by addition of *N* ammonia. To the solution, now at p_H 7·0, were added 5 cc. of alumina C_γ suspension. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. In all ten such adsorptions at p_H 7·0 were carried out upon the solution. This procedure removes the carboxypolypeptidase, and the solution from the final adsorption contained the proteinase.

This solution, which was used in the later experiments with kaolin, was kept in the ice-chest. Prior to treatment with kaolin, the proteinase content of the solution was determined by activating with enterokinase and measuring the resulting activity according to the method of Linderstrøm-Lang and Steenberg [1929]. It was found that the solution contained 0·95 enzyme unit per cc.

(*c*) *Adsorption of the proteinase upon kaolin.* Experiments were next carried out to determine whether the proteinase is adsorbed by kaolin, since in the experiments of Willstätter *et al.* [1926] it was found that "trypsin," under certain conditions, is adsorbed by kaolin.

To 30 cc. of the ice-cold proteinase solution at p_H 7·0 were added 5 cc. of a suspension of kaolin in distilled water, which contained 0·4 g. of B.D.H. kaolin *puriss.* The mixture was shaken and left for 10 minutes in the ice-chest, after which it was centrifuged. The clear centrifugate was treated again with 5 cc. of kaolin suspension and the above procedure repeated. This operation was carried out four times in all.

The next process was to attempt the elution of the proteinase from the kaolin and thus examine the degree of success obtained with the adsorption process. Accordingly the kaolin from the adsorption experiments was shaken up with 30 cc. of ice-cold solution E. (Solution E was made up of 57 parts by volume of 1 % diammonium phosphate solution, 3 parts by volume of *N* ammonia, and 40 parts by volume of glycerol-water solution—this solution having already been employed by Willstätter *et al.* [1926] in connection with trypsin.) The suspension of kaolin in solution E was kept in the ice-chest for 15 minutes and was then centrifuged. The clear liquid from the centrifuging was then neutralised with *N* acetic acid and kept in the ice-chest. The proteinase content of this solution was then examined, and it was found to be

0·78 enzyme unit per cc. That is, the process of adsorption on kaolin together with the subsequent elution may be considered quite satisfactory.

The heat-inactivation of the proteinase.

For the experiments in this section the starting material was the stock solution of proteinase remaining after the alumina adsorptions. Immediately before a run 30 cc. of this solution were treated with kaolin and the proteinase then eluted from the kaolin as described above. By varying the glycerol concentration in the solution employed for the elution, it was possible to examine the heat-inactivation of the proteinase in solutions differing in glycerol content, but identical in electrolyte concentration.

In the first place it was necessary to examine whether the heat-inactivation of the proteinase resembled that of "trypsin" in respect of the applicability of the unimolecular expression. The p_H measurements in this work were made by means of the glass electrode at room temperature. By way of illustration Table I gives the data obtained in aqueous solution.

Table I.

Temperature 50°. Glycerol 0 %. p_H 6·8.

Time (mins.)	Enzyme units per cc.	$k \times 10^3$
0	0·78	—
45	0·57	7·0
90	0·42	6·9
135	0·30	7·1

It is seen that there is agreement with the unimolecular expression. With solutions containing glycerol agreement with the unimolecular expression was also found.

The critical increment of the process at different glycerol concentrations.

The critical increment of the process was determined by measuring the value of k at 50° and at 60° and then substituting into the integrated form of the equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

The experiments were carried out with solutions containing 40, 20 and 0 % glycerol respectively. It was not feasible to work with a glycerol concentration greater than 40 %, because of the extreme difficulty in centrifuging the kaolin in solutions of higher glycerol content.

The results obtained are given in Table II.

Thus it is seen that the magnitude of the critical increment is sensibly independent of the glycerol concentration, lying between 35,000 and 40,000 calories per "molar unit" of enzyme.

Table II.

		Time (mins.)	Enzyme units per cc.	k	E (calories)
Glycerol 0 % p_H 6·9	Temp. 50°	0	0·80	$7·6 \times 10^{-3}$	39,000
		70	0·47		
	Temp. 60°	0	0·80	$4·7 \times 10^{-2}$	
		10	0·50		
Glycerol 20 % p_H 6·8	Temp. 50°	0	0·73	$3·6 \times 10^{-3}$	40,000
		90	0·53		
	Temp. 60°	0	0·72	$2·3 \times 10^{-2}$	
		15	0·51		
Glycerol 40 % p_H 6·9	Temp. 50°	0	0·76	$2·1 \times 10^{-3}$	35,000
		110	0·60		
	Temp. 60°	0	0·78	$1·1 \times 10^{-2}$	
		15	0·66		

Discussion.

From the results given above we may make comparisons, especially in relation to the results of other work carried out in this laboratory, which may be tabulated as follows.

1. The magnitude of the critical increment for heat-inactivation of the proteinase is practically identical with that found for trypsin-kinase, enterokinase and "trypsin." Thus the separation of the carboxypolypeptidase has not influenced quantitatively the thermal stability of the proteinase.

2. The critical increment, within the limits of experimental error, is independent of the glycerol concentration of the solution, and we may conclude by inference that this holds true for "trypsin."

3. The fact that the critical increment does not vary with the glycerol concentration is in agreement with the similar result found by McGillivray [1930] for purified lipase.

4. The magnitude of the critical increment approximates to that found by McGillivray for purified lipase, namely 46,000 calories.

Probably the most striking of these facts is the resemblance between the behaviour of proteinase, trypsin-kinase, enterokinase and that of lipase. Such a resemblance between enzymes differing so markedly in specificity as do lipase and proteinase indicates that, although heat-inactivation results in destruction of their specific active groupings, the primary effect must be upon the supporting "core" of each enzyme. Further, it seems reasonable to conclude that the cores of each enzyme must be very closely similar, either in chemical structure or in the physical state of their aggregation. Their difference in specificity may be governed by the spatial distribution of active groups on the surface of the core.

In general it will be observed that the values obtained by the writer and by McGillivray for the critical increment are less than those which have been found by previous workers, who have for the most part employed cruder enzyme preparations. McGillivray showed that the purification of lipase is accompanied by a drop in the value of the critical increment of heat-inactivation from 95,000 calories to 46,000 calories. Yet it might still be considered

that the magnitude, *circa* 40,000 calories, found for the proteinase and lipase is still influenced by the presence of adventitious material associated with the true core of the enzyme. It is clear, however, by considering the case of the proteinase, that such material is so firmly attached to the enzyme that it defies separation therefrom by methods of adsorption which have been successfully applied to separate enzymes which must be regarded as very analogous in their adsorption affinities. Thus such material, if present, may very well be regarded as an integral part of the enzyme core.

SUMMARY.

1. Experiments have been carried out upon the adsorption by means of kaolin of proteinase obtained in solution by the method of Waldschmidt-Leitz and Purr [1929]. It is found that the proteinase is removed from solution by the kaolin and may be successfully eluted therefrom. This additional procedure has been incorporated with the technique of Waldschmidt-Leitz and Purr to prepare solutions of the proteinase.

2. It is found that the heat-inactivation of the proteinase proceeds in agreement with the unimolecular expression.

3. The critical increment of heat-inactivation has been determined in solutions containing 40, 20 and 0 % glycerol. It is found that the critical increment is independent of the glycerol concentration, and is of the value of 35,000–40,000 calories per molar unit of enzyme.

4. Resemblances between the heat-inactivation of proteinase, "trypsin," enterokinase and purified lipase are discussed. It is suggested that in general heat-inactivation is primarily an effect upon the enzyme core. It is pointed out in addition that it seems reasonable to conclude that different enzymes must possess cores which are closely similar, either in chemical structure or in the physical state of their aggregation.

REFERENCES.

Linderstrøm-Lang and Steenberg (1929). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 16.
McGillivray (1930). *Biochem. J.* **24**, 891.
Pace (1930). *Biochem. J.* **24**, 606.
Waldschmidt-Leitz and Purr (1929). *Ber. deutsch. chem. Ges.* **62**, 2217.
Willstätter and Kraut (1923). *Ber. deutsch. chem. Ges.* **56**, 1117.
—— Waldschmidt-Leitz, Dunaiturria and Kunstner (1926). *Z. physiol. Chem.* **161**, 191.

CLXIII. AUTOLYSIS IN MALIGNANT AND NORMAL RABBIT TISSUES[1].

By HAROLD IDRIS PRICE.

From the Muspratt Laboratory of Physical and Electro-chemistry, University of Liverpool.

(*Received August 5th, 1931.*)

In connection with proteolytic change in malignant growths Robin [1919] arrived at the following conclusions, based upon an analysis of malignant and normal human tissue.

(1) There is "a decrease in the insoluble nitrogen and an increase in the soluble nitrogen in the 'relatively healthy' regions bordering upon the growth as compared not only with the most cancerous regions but also with normal healthy tissue."

(2) There is "an increase in the insoluble nitrogen, *i.e.* protein nitrogen, in the parts of the tissue which are most attacked, with a diminution in soluble nitrogen."

In other words the percentage ratio of protein nitrogen to total nitrogen is lower in the "relatively healthy" region than in either the growth or the normal tissue. A typical example of Robin's findings is as follows:

	$\frac{\text{Protein N}}{\text{Total N}} \times 100$
Most cancerous region	58·01
Relatively healthy region	47·01
Normal liver (after 26 hours' autolysis)	70·55

Robin considered that the most logical interpretation of these facts was that the proteins of a cancer are formed at the expense of the products of autolysis of the proteins of the organ in which it develops. This view implies that the tissue immediately surrounding the growth is abnormal with respect to autolysis, and the point is of such importance that it was considered worthy of further investigation.

In the present investigation nitrogen analyses were carried out using rabbit tissues—cancerous, adjacent normal tissue, and the normal tissue of the same organ.

Experimental.

The rabbit tissue used for the experiments was liver and kidney in which metastatic deposits of malignant growth were present, the rabbits having been

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation, Prof. W. Blair Bell, Director, University of Liverpool.

previously inoculated with the Brown-Pearce tumour. On removing the organ from the animal the growth was cut away from the rest of the organ and the adjacent normal tissue also removed from the rest of the normal tissue. A representative sample of each section was submitted to independent histological examination.

The tissue was first minced up as finely as possible with a pair of scissors and then weighed out in portions of 0·3–0·5 g. This is the smallest weight of tissue compatible with uniform samples. Six samples of each section of tissue were weighed out, two in combustion test-tubes for total nitrogen determinations, two in small glass tubes for protein nitrogen determinations and two in small tubes for dry weight estimations. The last two were heated at 105–115° for 36 hours in an electric oven. It was found that this drying was enough to give constant dry weights and the results could be reproduced to within 2 %.

Following the well-known micro-Kjeldahl technique [Pregl, 1924] for total nitrogen determination, there was added to each sample 1 cc. concentrated sulphuric acid, a knife-point of nitrogen-free copper sulphate and the same amount of potassium sulphate, and the mixture heated over a micro-burner until it charred. A few drops of 30 % hydrogen peroxide were then added and the mixture again heated to charring. This process was repeated until all the organic matter had disappeared, judging by the absence of charring. The time required is shortened considerably if before adding the sulphates the tissue is charred with concentrated sulphuric acid then heated for some time in a water-bath, a few drops of hydrogen peroxide being added from time to time. After the oxidation was complete the contents of the tube were diluted and made up to 25 cc. 2 cc. samples were taken and the nitrogen estimated in a micro-Kjeldahl distillation apparatus. Blank experiments were made with concentrated sulphuric acid, copper sulphate, potassium sulphate, distilled water and hydrogen peroxide.

Each sample for protein determination was thoroughly mixed with pure anhydrous sodium sulphate and allowed to harden. The hard mass was transferred to a small flask with absolute alcohol and boiled on a water-bath for an hour. The liquid was then centrifuged and the remaining mass extracted with boiling water slightly acidified with acetic acid. The extraction was continued until the extracting liquid showed no sign of sulphate. The remaining proteins were incinerated and the nitrogen estimated by the micro-Kjeldahl method as before.

Samples (in duplicate) were taken from tissue representing the three regions, *viz.* the growth, the adjacent normal tissue, and the normal tissue, and also duplicate distillations were carried out with each sample. The results obtained by the above method agree to within 2 %.

Results.

In all, tissues from 16 rabbits were analysed and the results are summarised in Table I.

Table I. *Analytical results.*

Rabbit No.		6	7	11	12	13	4	5	8	2	1	10	14	15	16	3	9
Description of tissue		K.	L.	L.	L.	K.	K.	L.	L.	K.	K.	K.	K.	L.	K.	K.	K.
P.M. period (hours)		0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Growth	Dry weight	18·9	21·4	21·8	22·3	20·4	15·5	22·7	21·3	21·2	20·5	18·1	22·0	24·8	20·6	20·3	20·9
	Total nitrogen	2·68	2·86	2·79	3·05	2·79	2·83	3·36	2·92	3·26	2·75	2·13	3·26	3·67	2·84	2·70	2·69
	Protein ,,	1·72	2·42	2·44	2·55	2·21	2·26	2·56	2·35	2·63	2·34	1·97	2·62	3·23	2·60	2·45	2·23
	Soluble ,,	0·96	0·44	0·35	0·50	0·58	0·57	0·80	0·57	0·63	0·41	0·16	0·64	0·44	0·24	0·25	0·46
Adjacent normal tissue	Dry weight	20·4	24·0	23·5	26·7	22·2	16·6	22·2	23·0	19·8	20·2	19·6	20·5	24·1	19·6	17·5	17·4
	Total nitrogen	3·03	3·25	2·98	3·48	3·66	2·27	3·17	3·39	3·15	2·72	2·59	2·88	3·76	2·78	2·38	2·44
	Protein ,,	2·27	2·90	2·70	3·00	3·24	1·79	2·45	2·89	2·45	2·02	2·22	2·20	2·97	2·30	2·10	1·96
	Soluble ,,	0·76	0·35	0·28	0·48	0·42	0·48	0·72	0·50	0·70	0·70	0·37	0·68	0·79	0·48	0·28	0·48
Normal tissue	Dry weight	19·9	—	22·6	25·2	21·8	15·9	23·5	25·1	18·6	—	15·9	22·9	24·2	18·2	15·9	17·9
	Total nitrogen	2·74	—	2·56	3·73	3·44	2·25	3·08	3·51	2·81	2·80	1·96	2·85	3·68	2·30	2·14	2·41
	Protein ,,	2·13	—	2·48	3·61	3·20	1·76	2·70	3·09	2·35	2·62	1·83	2·53	2·92	2·15	1·91	2·05
	Soluble ,,	0·61	—	0·08	0·12	0·20	0·49	0·38	0·50	0·46	0·18	0·13	0·32	0·39	0·15	0·23	0·36

Percentage ratios of protein nitrogen to total nitrogen

P.M. period	0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Growth	64·2	84·6	87·5	83·6	79·2	79·9	76·2	80·5	80·7	85·1	92·5	80·4	88·0	91·6	90·7	82·9
Adjacent normal tissue	67·3	89·2	90·6	86·2	88·5	78·9	77·3	85·3	77·8	74·3	85·7	76·4	79·0	82·7	88·2	80·3
Normal tissue	77·7	—	96·9	96·7	93·1	78·2	87·7	88·0	83·6	93·6	93·4	88·8	79·4	93·5	89·3	85·1

In the second horizontal column the letters K. and L. represent kidney and liver respectively.

The *post mortem* period, represented by P.M. period, is the time elapsing between the death of the animal and the excision of the tissue.

The "dry weight" is the percentage ratio of the weight of dried tissue to the weight of the tissue before drying, *i.e.* fresh tissue.

The nitrogen figures represent, in all cases, the percentage weight of nitrogen in fresh, *i.e.* wet tissue.

At the foot of Table I are given the percentage ratios of protein nitrogen to total nitrogen in the growth, adjacent normal tissue and normal tissue.

Discussion of results.

In the first place it is necessary to draw attention to the conditions under which Robin's results were obtained. Robin worked with human liver, stomach and pancreas which were obtained *post mortem*. As a consequence the tissue was not excised from the corpse until 24–27 hours after death. As will be seen later this fact probably accounts for the results that Robin obtained. The normal tissue employed by Robin was tissue from a healthy person killed in an accident. In the present investigation the normal tissue was taken from the same organ as the growth and consequently probably gives a more useful comparison than that of Robin. It follows that Robin's results and the group with P.M. period of 12 hours in the present investigation ought to be comparable.

The results obtained by the writer indicate fundamental differences according to the P.M. period of the tissue employed. In the group with P.M. period less than 3 hours the percentage ratio of protein nitrogen to total nitrogen is greatest in the normal tissue, lowest in the growth, with an intermediate value in the adjacent normal tissue. When the P.M. period is 12 hours the ratio is lower in the adjacent normal tissue than in either the normal tissue or the growth—this might for convenience be known as the "Robin effect." When the P.M. period exceeds 12 hours this "Robin effect" seems to be less pronounced. Hence if the tissue is analysed when it is as near as possible to the condition obtaining *in vivo*, *i.e.* P.M. period less than 3 hours, there is no unexpected proteolytic condition in the adjacent normal tissue. The "Robin effect" manifests itself after a *post mortem* period of 12 hours for rabbit tissue. Hence this effect is probably due to *post mortem* changes and does not represent conditions of malignancy *in vivo*.

At first sight this minimum value of the ratio protein nitrogen to total nitrogen in the adjacent normal tissue after a P.M. period of 12 hours seems to be explained by assuming that autolysis takes place to the greatest extent in this region. This can be shown not to be the case by consideration of Table II. The second column gives the percentage values of protein nitrogen in the adjacent normal tissue, and the third column gives the percentage

values of protein nitrogen in the normal tissue calculated on the basis of the same dry weight as the corresponding adjacent normal tissue.

Table II.

Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue	Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue
2	2·45	2·50	10	2·22	2·26
3	2·10	2·10	11	2·70	2·58
4	1·79	1·84	12	(3·00)	(3·72)
5	2·45	2·55	13	3·24	3·21
6	2·27	2·18	14	2·20	2·26
8	2·89	2·83	15	2·97	2·91
9	1·96	1·99	16	2·30	2·32

It will be seen that in each case, whatever the P.M. period, the values in columns 2 and 3 are the same within experimental error. This shows that autolysis is not occurring at an excessive rate in the adjacent normal tissue. Incidentally the above figures show that the growth does not, *in vivo* or *post mortem*, liberate proteolytic enzymes into the adjacent tissue.

In an attempt to understand these *post mortem* changes the figures giving the concentration of soluble nitrogen in the tissues may also with advantage be classified according to the P.M. period of the tissue:

Table III. *Concentration of soluble nitrogen.*

(% N per weight of water in tissue.)

	Rabbit No.	P.M. period	Growth	Adjacent normal tissue	Normal tissue
Kidney	4	1	0·68	0·58	0·58
,,	6	0	1·18	0·96	0·76
,,	13	0	0·73	0·54	0·26
Liver	5	1	1·04	0·93	0·49
,,	7	0	0·56	0·46	—
,,	8	3	0·72	0·65	0·67
,,	11	0	0·45	0·37	0·10
,,	12	0	0·64	0·66	0·16
Kidney	2	5	0·80	0·87	0·57
,,	1	12	0·52	0·88	—
,,	10	12	0·20	0·46	0·16
,,	14	12	0·82	0·86	0·42
,,	16	12	0·15	0·48	0·24
Liver	15	12	0·59	1·04	1·00
Kidney	9	24	0·58	0·58	0·43
,,	3	24	0·30	0·34	0·27

It will be noticed that when the P.M. period is less than 3 hours the concentration of soluble nitrogen in the growth tends to be greater than that in the adjacent normal tissue which in turn tends to be greater than in the normal tissue.

When the P.M. period is 12 hours the concentration of soluble nitrogen seems to reach a maximum in the adjacent normal tissue. This is also the case in Robin's experiments on human tissue 24–27 hours *post mortem*.

When the P.M. period is 24 hours there seems to be a tendency for the concentration of soluble nitrogen to be equal in all three tissue regions.

From these figures it might be assumed that *in vivo* the concentration of soluble nitrogen is highest in the growth, becoming lower in the adjacent tissue and lower still in tissue further away from the growth. Hence on death it is possible that some controlling factor, such as the degree of permeability of the membranes, is altered and the soluble nitrogen travels from the place of highest osmotic pressure, *i.e.* the growth, to lower osmotic pressure regions. Thus in process of time soluble nitrogen will be transferred from the growth in the first instance to the adjacent normal tissue. This will mean that in the growth the total nitrogen will be lower while the protein nitrogen remains the same, that is the ratio of protein nitrogen to total nitrogen will be higher than *in vivo*. Also the adjacent normal tissue will increase in total nitrogen while the protein nitrogen is unaltered, so that the ratio of protein nitrogen to total nitrogen will be lower than *in vivo*. This is the condition we have called the "Robin effect."

The maximum concentration of soluble nitrogen in the adjacent normal tissue after 12 hours *post mortem* is rather puzzling but it is possibly due to some property of the tissue membranes. After 24 hours the membranes may be regarded as having become completely permeable and the concentration of soluble nitrogen tends to be the same in each of the regions.

In Table I all the percentages are referred to fresh (wet) tissue. Additional evidence in support of *post mortem* diffusion causing the different effects is however afforded by an examination of the dry weights of the tissues. From Table IV it will be noticed that when the P.M. period is less than 3 hours the

Table IV. *Dry weights.*

	Rabbit No.	P.M. period	Normal tissue	Growth
Kidney	4	1	15·9	15·5
,,	6	0	19·9	15·5
,,	13	0	21·8	20·4
Liver	5	1	23·5	22·7
,,	7	0	—	21·1
,,	8	3	25·1	21·3
,,	11	0	22·6	21·8
,,	12	0	25·2	22·3
Kidney	2	5	18·6	21·2
,,	10	12	15·9	18·1
,,	1	12	—	20·5
,,	14	12	22·9	22·0
,,	16	12	18·2	20·6
Liver	15	12	24·2	24·8
Kidney	9	24	17·9	20·9
,,	3	24	15·9	20·3

growth tends to contain more water than the normal tissue. This is the usual behaviour of rapidly growing tissues. However when the P.M. period is 12 hours and over, the position is reversed and the normal tissue tends to be greater in water content than the malignant region. Hence, after death, water

begins to disappear from the growth into the surrounding organ. This movement of water makes it very probable that soluble nitrogen is also transferred throughout the organ after death.

The evidence against the Robin view that the normal tissue adjacent to a malignant growth is abnormal *in vivo* in a proteolytic sense is the following:

(*a*) the histological examination of normal tissue and adjacent normal tissue does not indicate any difference between them;

(*b*) in both regions the protein nitrogen content is the same when calculated on the same basis with respect to water content;

(*c*) when the P.M. period is less than 3 hours the ratio of protein to total nitrogen in the adjacent normal tissue is intermediate between the values for the growth and the normal tissue and not below both of them.

Summary.

Malignant, adjacent normal, and normal rabbit tissues have been analysed for total nitrogen, protein nitrogen and soluble nitrogen. The results obtained show that Robin's [1919] view that in malignancy, normal tissue is autolysed and that the growth builds up its proteins from the autolytic products is not correct. The "Robin effect" has been obtained by allowing the tissue to remain in the body for 12 hours after death before excision. This effect has been shown to be due probably to *post mortem* diffusion in the organ. The "Robin effect" does not manifest itself in the case of freshly examined tissue and is therefore not operative *in vivo*.

Evidence has been obtained to the effect that after death water disappears from the growth into the surrounding tissue.

Evidence has also been obtained that a malignant growth does not liberate proteolytic enzymes into the surrounding tissues.

The writer wishes to acknowledge his indebtedness to Dr M. M. Datnow for the supply of rabbit tissues and also for the histological examination of the tissues.

REFERENCES.

Robin (1919). *Bull. Acad. Med.* **81**, 799.

Pregl (1924). Quantitative organic analysis (translated by E. Fyleman from the second German edition).

CLXIV. THE ACID-DENATURATION OF HAEMOGLOBIN.

By NORMAN URQUHART MELDRUM[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 15th, 1931.*)

The change undergone by proteins in aqueous solutions when subjected to the action of various influences, such as those of acid, alkali, heat, light and alcohol, is known as "denaturation." Acids and alkalis produce metaprotein, a product soluble in acids and alkalis, but, in the absence of salts, insoluble at the isoelectric point. Boiling an isoelectric suspension of metaprotein converts it into an insoluble product like that obtained from native isoelectric protein under the influence of heat, alcohol, or shaking.

The coagulation of haemoglobin has been studied by Chick and Martin [1910], who used heat as denaturant: they found denaturation to take place only in the presence of water, and to follow the unimolecular law. They found the reaction to be characterised by the temperature coefficient of 1·3 per 1°, a value confirmed by Hartridge [1912], and by Lewis [1926]. Lewis obtained evidence that not only did H^+ and OH^- ions catalyse the reaction, but that water also entered into it, as a reactant rather than as a catalyst. He confirmed the observation of Chick and Martin that under certain specified conditions salts decrease the rate of denaturation, and his work was extended by Cubin [1929], who found that formaldehyde prevented the flocculation of denatured haemoglobin at p_H values greater than 7.

Wu [1929] distinguishes between "denatured" and "coagulated" protein. He regards them as chemical species distinct from each other. "Coagulation" occurs when protein is heated at the isoelectric point or when it is subjected to dehydrating agents, and involves a decrease in the number of free amino- and carboxyl-groups. Such condensed protein is rather insoluble in acids and alkalis. "Denatured" protein is obtained by the action of acids and alkalis, especially on warming, and denaturation consists, according to Wu, in the splitting of the protein with liberation of free $—NH_2$ and —COOH groups. Such denatured protein is much more soluble in acids and bases than is the condensed material.

The action of alcohol upon methaemoglobin is to give a substance which has lost the power of combining with oxygen [Arnold, 1900]. It was studied by Van Klaveren [1901] under the name of kathaemoglobin, and is now called

[1] Beit Memorial Research Fellow.

parahaematin [Keilin, 1926]. Alkali yields a similar product [Adair, unpublished observation quoted by Keilin, 1926], as do many other reagents, and it is now clear that parahaematin is a compound between denatured, or condensed, protein and oxidised haematin [Keilin, 1926]. Reduction gives the haemochromogen of Bertin-Sans and de Moitessier [1893] and of Anson and Mirsky [1925].

In general the denaturation of proteins has been regarded as irreversible. Though Michaelis and Rona [1910] report the heat-coagulation of electrolyte-free serum-albumin to be reversed by the action of alkalis, an observation confirmed by Spiegel-Adolf [1926], no like case appeared to be known, either with regard to condensed or denatured proteins, until Anson and Mirsky [1925] stated that the acid-denaturation of haemoglobin was essentially reversible. When a solution of haemoglobin was acidified and the mixture neutralised, complete precipitation resulted. By the action of alkalis on this coagulum, especially in the presence of cyanide, native haemoglobin could be isolated, best as the carbon monoxide derivative. It may be urged that some native material is carried down by the denatured protein on flocculating. Anson and Mirsky advanced two arguments against this: the yield of denatured reversed protein was about 70 %—yet when entirely denatured protein was precipitated in the presence of native, the latter was undiminished in concentration. Secondly the temperature of denaturation had no effect on the yield of reversed material obtained.

The problem has a dual nature. In the first place haemoglobin behaves as a typical protein, denatured or native. If its denaturation be reversible then it appears probable that the denaturation of other proteins may be so likewise. In the second place this phenomenon has bearings on the haemoglobin system itself. Anson and Mirsky [1925] regard haemoglobin and haemochromogen as forming an equilibrium, the point of balance being decided by the p_H. At strongly acid p_H values haemochromogen predominates in the equilibrium,

$$\text{haemoglobin} \rightleftharpoons \text{haemochromogen},$$

while near neutrality the protein is mainly haemoglobin.

In later experiments they have shown [1930] that the prosthetic group plays no decisive part in affecting reversibility. It is possible to cause the reversion of the denaturation of free globin in the complete absence of haematin. Yet the haematin moiety does play some part in the phenomena observed. Thus Wu and Lin [1927] observed that the amount of reversed protein was greater in the ratio of 1·8 to 1 when the initial protein was reduced haemoglobin in place of oxyhaemoglobin. The haemoglobins of various species appear to behave differently. When a solution of ox haemoglobin is acidified with acid, in the ratio of 4 cc. $N/5$ HCl to 6 cc. 10 % haemoglobin, and neutralised after at least 10 minutes, complete precipitation results, the colour of the coagulum being brown like that of acid haematin. After standing for a few minutes the colour changes to the red of parahaematin, and methaemo-

globin passes into solution. With horse haemoglobin the effects are different. The brown colour of the coagulum changes to the pink of parahaematin, but solution of methaemoglobin does not take place so readily, and the alkaline cyanide reagent of Anson and Mirsky must be used for native material to be obtained in the soluble state. The parahaematin of the haemoglobin of the horse seems to be stable over a wider p_H range than that of the ox (personal communication of Mr R. Hill).

Experimental.

When a solution of oxyhaemoglobin is acidified, changes take place in the system: acid methaemoglobin is formed if the p_H be not too low, and the solution contains free native and free denatured globin, as well as acid haematin. The analysis of such a system is complex; the first phenomenon studied was the formation of acid haematin from acid methaemoglobin.

In the majority of these experiments a solution of methaemoglobin was used. Ox blood corpuscles were washed with isotonic saline four times, laked with an equal volume of distilled water, and the stromata removed with alumina cream. Thereafter the procedure was identical with that advocated by Stadie and Ross [1926] for the electrodialysis of haemoglobin, except that the protein was first converted with 10 % ferricyanide to methaemoglobin.

The buffers employed throughout are those of McIlvaine (0·1 *M* citric acid and 0·2 *M* alkaline phosphate). The buffering power of these buffers is approximately linear throughout the p_H range used here, from p_H 2·2 to 4·8.

A titration curve of each methaemoglobin solution used was constructed by adding HCl in known amount and determining the p_H by the quinhydrone electrode. At the p_H values concerned this does not appear to denature the proteins. Thus the p_H values were not appreciably affected on allowing the solutions to stand with quinhydrone as they would have been had denaturation occurred progressively. A similar titration curve was obtained for gum arabic in 8 % solution. From these curves the amount of acid needed to bring the protein and gum arabic solutions to any p_H could be calculated roughly.

The experiments were carried out as follows. To buffer solution in a standard flask was added the solution of methaemoglobin to be investigated. The buffering power of the protein was overcome by adding acid in the amount calculated from the titration curves referred to. The final p_H was thus that of the buffer. It was found to be a matter of indifference here whether the acid was run into the solution before, or after, addition of the protein solution. The acid solution of protein thus obtained then contained acid methaemoglobin and acid haematin. The relative amounts of these substances were then estimated. This was done by use of the double-wedge trough described by Hill [1929].

In these experiments on acid methaemoglobin and acid haematin, the standard solutions put in the trough, one in each side, consisted of acid

methaemoglobin at p_H about 5·8[1], and acid haematin in 4 % gum arabic solution, dissolved in McIlvaine buffer at the p_H of the acid mixture of methaemoglobin and acid haematin to be analysed. Ten or twenty readings were then taken at the positions where the spectrum of the standards matched in all respects that of the solution analysed. Two sources of error militate against accuracy. The degree of dispersion affects the absorption [Keilin, 1926], and the standard contains no parahaematin. The latter effect can be of small importance, as the absorption of parahaematin is slight, and as it can only be in low concentration. In studying the formation of acid haematin from acid methaemoglobin the author has accepted the view of Keilin that the former indicates a physical state, namely a colloidal suspension of haemin protected by protein or gum arabic, rather than a definite chemical individual.

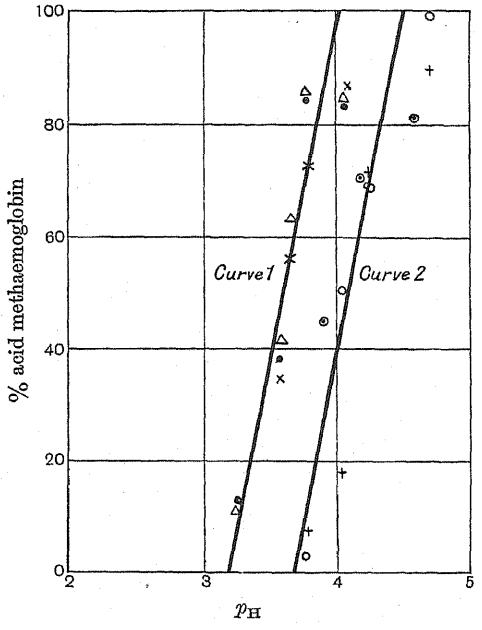


Fig. 1. The formation of acid haematin from acid methaemoglobin.

Curve 1. 2 cc. (approx.) 5 % solution of electrodialysed ox methaemoglobin+12·5 cc. of McIlvaine buffer+12·5 cc. water. 0·1 *N* HCl was then added, in amount sufficient to overcome the buffering of the protein and the volume diluted to 50 cc. The standards were acid methaemoglobin at a p_H of 5·5 in water, and a solution of acid haematin in 4 % gum arabic and McIlvaine buffer (12·5 cc. in 50 cc. total volume) at the p_H of the solution studied.

Each different type of point ×, Δ, and ●, refers to separate experiments on the protein.

Curve 2 was carried out with a different sample of protein.

Points + were obtained with 40 cc. of McIlvaine buffer in 50 cc. aqueous solution, ○ points with 20 cc. buffer in 50 cc. total volume. A third sample of protein has been studied. Its behaviour is shown by points ⊙. The experiments were carried out in McIlvaine buffer undiluted.

Curves 1 and 2 (Fig. 1) show formation of acid haematin from acid methaemoglobin as a function of p_H. Two different protein solutions have been studied. The results are similar but the curves do not coincide. On the

[1] It is not possible to have the methaemoglobin standard at the p_H of that to be analysed, for under such conditions it would dissociate to acid haematin and become denatured.

other hand, separate estimations on any one protein preparation agreed admirably, as can be seen from Fig. 1. Solutions of methaemoglobin brought to a p_H of 3·3 or 3·5 in McIlvaine buffer precipitate completely, as though fully denatured, when made isoelectric.

The protein solutions studied in curves 1 and 2 were treated with the alkaline cyanide reagent of Anson and Mirsky, and allowed to stand at a p_H of about 7·8 for 3 hours. Then the solutions were half saturated with ammonium sulphate and the precipitated protein filtered off. On reducing with sodium hydrosulphite and passing in carbon monoxide, the spectrum of carboxyhaemoglobin became visible. The whole was filtered after standing overnight. The total amount of the "reversed" protein was then estimated in the double-wedge trough. The standard solutions were carboxyhaemoglobin in one wedge and water in the other. The yields of native reversed protein obtained from the protein solutions which gave Fig. 1 are plotted in Fig. 2. It seems, that though there appears to be some acid methaemoglobin in

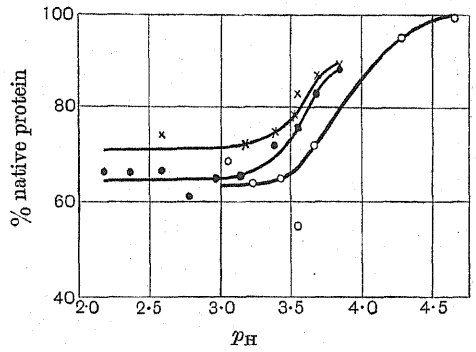


Fig. 2. Amount of carboxyhaemoglobin obtained from acid protein after the treatment with alkaline cyanide advocated by Anson and Mirsky.

The points have the same meaning as in Fig. 1.

solutions which precipitate entirely on neutralisation, yet this acid methaemoglobin is insufficient in amount to account for the yield of "reversed" protein obtained. This discrepancy is tabulated in Table I. It is perhaps due to the dissociation to acid haematin and free native globin. As the properties of protein solutions change rapidly with time, it is a matter of some difficulty to obtain sufficient data in the course of a few days from one solution of protein to provide a comparison of the amounts of acid methaemoglobin present and reversed protein obtained.

The behaviour of acid protein on neutralisation is worthy of closer examination. When a solution of methaemoglobin is brought to p_H 2·5, say, and then neutralised, all the protein is precipitated, and on redissolving the coagulum in the minimum amount of alkali and neutralising the alkaline solution as rapidly as possible, all the protein is again reprecipitated, and on filtering the coagulum only a small fraction of the total protein comes with the filtrate.

Table I.

p_H	Acid methaemoglobin estimated in double-wedge trough: Exp. 1 %	Exp. 2 %	Exp. 3 %	Native protein estimated in double-wedge trough as carboxyhaemoglobin: Exp. 2 %	Exp. 3 %	Value of R: Exp. 2 %	Exp. 3 %
			Protein preparation No. 1				
4·57	91·5	93·8	92	86·7 (?)	—	0·0	0·0 (?)
4·03	85	84	86	82	88·3	4·1	14
3·81	86	85·7	71·9	88	88·3	29·7	29·1
3·66	65·3	30·4 (?)	56·9	83·4	89·1	44·9	34·9
3·56	43·2	38·5	35	73·1	86·6	53·2	60
3·32	12·2	12·6	11·4	73	79·8	83·5	84·5
3·19	0	0	0	64·8	77·2	—	—
2·94	0	0	0	64·2	76·3	—	—
2·76	0	0	0	60·7	—	—	—
2·60	0	0	0	64·6	74·9	—	—
2·43	0	0	0	66	76·6	—	—
2·21	0	0	0	66	77·2	—	—
			Protein preparation No. 2			Mean values of R given here	
4·62	88·6	100·0	—	100	—	0·0 %	
4·24	71·7	69·1	—	95	—	26·4	
4·01	17 (?)	50·8	—	95	—	47·4	
3·66	—	3·3	—	73·5	—	92·0	
3·56	9·2	0	—	54·9	—	—	
3·46	0	0	—	66	—	—	
3·30	0	0	—	63·3	—	—	
3·10	0	0	—	68	—	—	
2·61	0	0	—	—	—	—	

Here R is given by the ratio

$$\frac{(\text{Total native HbCO} - \text{acid methaemoglobin}) \times 100}{\text{Total native HbCO}}.$$

The yield of "reversed" HbCO is plotted in Fig. 2, the amount of acid methaemoglobin in Fig. 1, curves 1 and 2.

If, on the other hand, the alkaline solution of protein is allowed to stand for some minutes and then neutralised with acid and filtered, a large amount is now found to be soluble. In other words the protein has "reversed" in the alkaline solution. When the protein solution is reduced with sodium hydrosulphite and treated as above, the haemoglobin is found to "reverse" more rapidly than when no reduction occurs. The following data illustrate the behaviour of methaemoglobin.

Into three 25 cc. graduated flasks were run 6·25 cc. McIlvaine buffer, p_H 2·9, and an equal volume of water, 0·5 cc. of protein solution, and 0·87 cc. of 0·1*N* HCl. Each flask was allowed to stand for some time at the acid p_H, flasks 1 and 2 for 8 minutes, flask 3 for 25 minutes. (Time is a non-variant in such a system, and does not affect the final result.) At the end of the times concerned the protein solutions were neutralised with NaOH and the precipitate redissolved in the minimum amount of alkali and immediately neutralised with acid and filtered. The protein in the filtrate was then estimated as alkaline methaemoglobin. The solution in flask 3 was allowed to stand for 10 minutes in alkali before neutralisation with acid and filtration. Then the

dissolved material was estimated as with flasks 1 and 2. The results are set out below.

Flask No. ...	1	2	3
Time kept alkaline	0 mins.	0 mins.	10 mins.
Alkaline methaemoglobin	10 % (?)	0 % (?)	64 %

It is thus clear that for "reversion" to take place the protein must stand for some time in alkaline solution. At the end of this time soluble protein is obtained which shows a spectrum apparently identical with that of alkaline methaemoglobin. When the system is studied spectroscopically the spectrum of alkaline methaemoglobin is visible from the beginning. A solution of methaemoglobin treated exactly as before shows, on spectroscopic examination when in alkaline solution, the spectrum of alkaline methaemoglobin, even though it can be entirely precipitated on adding acid. This spectrum seems to exist initially at the same intensity as when the protein is entirely reversed. Thus the change in solubility occurring when the protein stands in alkali is not paralleled by any corresponding change in spectrum. If spectroscopic evidence be accepted as a criterion of denaturation, then native protein exists as alkaline methaemoglobin, even though this can be completely precipitated on neutralisation. Similarly the spectrum of reduced haemoglobin can be seen in such solutions and also that of carboxyhaemoglobin.

That no evident change in spectrum occurs on standing is shown by the following data.

In each of two 50 cc. graduated flasks were put 12·5 cc. of McIlvaine buffer p_H 2·21 and 2·47 respectively, 3·8 and 2·8 cc. of 0·1 *N* HCl and water to make the final volume 25 cc. Then 1 cc. of methaemoglobin solution was run in, and the protein allowed to stand for 30 minutes in acid solution. After this the solution was neutralised, the coagulum dissolved in the minimum alkali required, and the spectrum studied.

A series of 10 readings was then taken over a short range of time, the final reading being about 6 minutes after the first, and the composition of the protein mixture calculated from the mean of each adjacent two. The standards in the double-wedge trough were alkaline methaemoglobin in one side and an alkaline solution of parahaematin in the other. The results are tabulated below.

Reading No. ...	1	2	3	4	5
			p_H 2·21		
% alkaline methaemoglobin	64	62	66	66	67
			p_H 2·47		
% alkaline methaemoglobin	67	64	64	65	67

These values are, however, precisely those which would be obtained on "reversion" of the protein according to the method of Anson and Mirsky.

When the protein solution is reduced the spectrum of reduced haemoglobin can be seen, and, like alkaline methaemoglobin, reduced haemoglobin appears

to exist under those conditions in a solution which is entirely precipitated on bringing to the isoelectric point. The same holds for carboxyhaemoglobin.

When a study is made of the spectrum shown by haemoglobin solution brought originally to an acid p_H and then to p_H 7·8 to 8, it is found that the spectrum varies according to the p_H to which the protein was brought initially. Fig. 3 shows the relationship between the amount of alkaline methaemoglobin estimated spectroscopically and the initial p_H of the solution before making alkaline. The abscissae refer, not to the p_H of the protein solution during the time of estimation, but to the p_H of the McIlvaine buffer at which denaturation took place. As has been before mentioned the amount of native protein as estimated in this way seems independent of the solubility of the protein at the isoelectric point, *i.e.* of the length of time the protein has stood in alkali at p_H about 8. The details of these experiments are as follows. After having made the protein solution acid in precisely the manner described for the experiments with acid methaemoglobin and acid haematin, alkali was added in amount just sufficient to redissolve the precipitated protein. A clear solution was obtained which showed the spectrum of alkaline methaemoglobin. This was placed in the cell of the double-wedge trough and estimated against standards of alkaline methaemoglobin and an alkaline solution of parahaematin. Table II gives some numerical data obtained in this way.

Table II.

p_H	Alkaline methaemoglobin %	Carboxyhaemoglobin in alkaline solution %	Carboxyhaemoglobin in acid solution %
4·57	—	—	104
4·03	—	—	—
3·81	—	88·3	105
3·66	—	89·1	87·5
3·56	—	86·6	85·7
3·32	—	79·8	74
3·19	—	77·2	77·9
2·94	—	76·3	77·5
2·76	—	79	73
2·60	—	74·9	(?)
2·43	—	76·6	(?)
2·21	—	77·2	(?)
4·62	100	100	—
4·24	—	95	—
4·01	65·3	—	—
3·66	53·5	73·5	—
3·56	51·5	54·9	—
3·46	57·2	66	—
3·30	—	63·3	—
3·10	55·2	68	—
2·61	47·5	—	—
4·20	90·1	—	—
4·05	74	—	—
3·78	72	—	—
3·56	76	—	—
3·33	64·2	—	—
3·12	63·2	—	—
2·90	64	—	—

Similar curves have been obtained by bringing solutions of methaemoglobin to a series of definite p_H values in McIlvaine buffer and then "reversing" the solutions according to the method of Anson and Mirsky. This involves the treatment of the solution with alkaline cyanide reagent for a few hours, the p_H being about 8, followed by the separation of insoluble protein with half saturated ammonium sulphate, reduction of the filtered solution with sodium hydrosulphite and saturation with carbon monoxide. The reversed protein was estimated as carboxyhaemoglobin in the double-wedge trough, the standards being carboxyhaemoglobin and water. Table II shows the data obtained thus, and Fig. 3 illustrates the similarity between

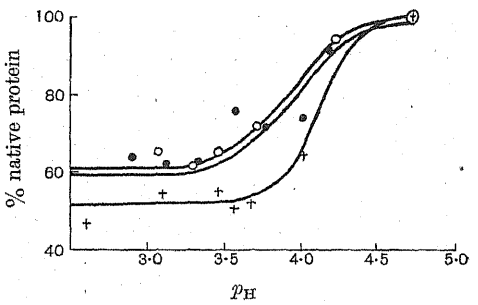


Fig. 3. A comparison of protein estimated as alkaline methaemoglobin after having been made acid in buffer, with that obtained as carboxyhaemoglobin after the cyanide treatment of Anson and Mirsky.

In order to obtain the solution of alkaline parahaematin the solution of haemoglobin was brought to a p_H of about 3 in phthalate buffer (Clark), and about 2·5 cc. N HCl added per 5 cc. phthalate buffer; the whole was warmed to about 60° and allowed to stand for about ½ hour, after which it was neutralised and made slightly alkaline. Points +, ● show the behaviour of protein so treated, while points ○ show the native material obtained after "reversion."

the amount of "reversed" protein and that estimated as alkaline methaemoglobin in the manner previously described. It is clear that the two curves coincide closely.

The problem may be attacked in a slightly different manner. When an acid solution of protein, such as has been studied above, is reduced with sodium hydrosulphite and aerated, the spectrum of oxyhaemoglobin can be seen. The bands rapidly disappear owing to the instability of the molecule at acid p_H. Partial neutralisation makes for greater stability, but it was never found possible to estimate the oxyhaemoglobin. When an acid solution of protein is reduced with sodium hydrosulphite in the presence of CO a solution is obtained which can be entirely precipitated on neutralisation. The solution shows a spectrum apparently identical with that of carboxyhaemoglobin, provided the p_H of the acid solution is not too low, when the spectrum of CO-reduced haematin appears; and on estimating the substance which resembles carboxyhaemoglobin against a standard of carboxyhaemoglobin in the double-wedge trough, a curve is obtained which coincides between p_H 4 and 5 with those obtained by reversion and as alkaline methaemoglobin. The spectrum of carbon monoxide-haemochromogen is very similar to that of

carboxyhaemoglobin and cannot be distinguished from it by the optical methods here used. Were, however, the protein entirely denatured and the substance estimated entirely CO-haemochromogen, it is hard to see how the amount of haemochromogen could vary (see Fig. 4) as no acid CO-reduced haematin could be observed at any p_H less than about 2·8. Before estimating the native protein the solutions were aerated thoroughly in each case in order to oxidise any haemochromogen present.

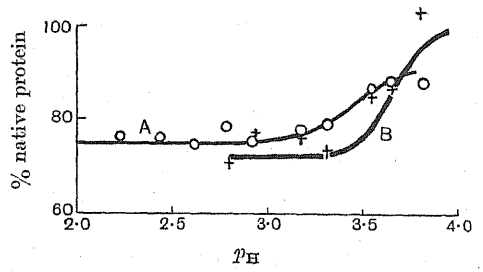


Fig. 4. Comparison of carboxyhaemoglobin obtained after treatment with alkaline cyanide with that estimated at acid p_H values down to p_H 2·75.

Points ○ refer to values obtained by making acid in McIlvaine buffer (12·5 cc. buffer in 50 cc. total volume), neutralising with $N/5$ NaOH and dissolving in about 5 cc. of cyanide solution, adding an equal volume of sat. $(NH_4)_2SO_4$ and filtering before converting to HbCO.

Points + were obtained by comparing the acid solution of protein with standards of carboxyhaemoglobin. The abscissae have different meanings for the two curves. For curve A the abscissae refer to the p_H to which the protein was brought in buffer, and for curve B to the p_H at which the estimation was carried out.

Table III gives data obtained in a series of preliminary experiments. The protein was about 5 % in concentration. To 1 cc. of this protein was added $N/5$ HCl in the amounts tabulated. Different volumes of $N/5$ NaOH were then added to this acid solution, reduction effected by adding $Na_2S_2O_4$, and then CO was passed in. The reduced solutions were then studied in the double-wedge trough, the standard solutions being carboxyhaemoglobin and water.

Table III.

cc. $N/5$ HCl added per 1 cc. protein	cc. $N/5$ NaOH added to acid mixture	% HbCO	cc. $N/5$ NaOH added	% HbCO
10	8·5	71·7	10	85
10	9·5	—	8·5	81
10	9·5	—	9·5	73·4
10	15·0	76·4	—	—
10	10·0	75·5	12·0	—
10	10·0	79·0	12·0	78·0
5	5·0	75·0	4·75	70·0
5	4·5	64·0	4·75	62·0

Where excess alkali was added the reduction was carried out by means of Stokes's reagent (alkaline reduction in 10 % sodium tartrate and dilute ferrous sulphate). The existence of HbCO at strongly acid p_H having been shown to be likely, the next series of experiments consisted in measuring the amount of HbCO obtainable between p_H 2·2 and 4·7. The curve thus obtained (Fig. 4) coincides closely with that obtained on reversion of the acid protein. Table II

compares the yield of native protein, obtained after reversion by the method of Anson and Mirsky, with the amounts estimated in the double-wedge trough at acid p_H as HbCO, and with the protein estimated as alkaline methaemoglobin.

Peculiarities in the behaviour of haemoglobin.

Chick and Martin found the temperature coefficient of the denaturation of haemoglobin to be 1·3 per 1°. Temperature, however, has little effect on the yield of native protein by reversion.

10 cc. $N/5$ HCl was added to 6·5 cc. 10 % oxyhaemoglobin. After 3 minutes the whole was neutralised with $N/5$ NaOH. The coagulum was washed with water and dissolved in KCN-buffer. The system was then reduced with $Na_2S_2O_4$ and CO bubbled through. Estimation of the amount of native protein showed that the effect of temperature is very small, the yield being about 43 % when the denaturation was carried out at 0°, and 32 % when at 40°.

The length of time the protein is kept at an acid p_H has no effect on the percentage recovery after the first minute when the temperature is 40°. This absence of effect of time is shown whatever the p_H; thus the amount of denatured protein does not increase on standing at, say, p_H 3·5 to the value obtained rapidly for p_H 2·8. There is no progressive increase on standing at acid reactions. The method used was as follows.

4 % ox methaemoglobin was acidified as in the previous experiment at a temperature of 40°. After definite intervals of time $N/5$ ammonia was run in. The precipitate was then dissolved in 2 % ammonia and reduced with Stokes's reagent. Aeration produced oxyhaemoglobin, which was estimated spectrophotometrically as in the case of other derivatives of haemoglobin. Fig. 5 illustrates the results obtained. Ox, sheep, and horse haemoglobins behaved

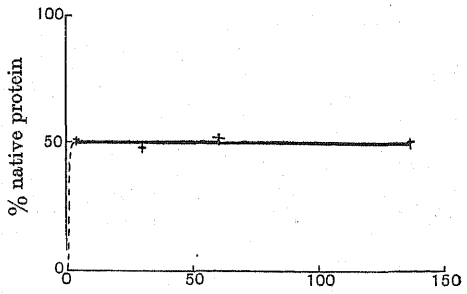


Fig. 5. Time-denaturation curve.

55 cc. 4 % ox methaemoglobin were acidified with 6·5 cc. $N/5$ HCl and $N/5$ ammonia was run in after various intervals.

alike in showing no progressive increase in denaturation on standing for various lengths of time at acid reaction at 0°, 20°, and 40°.

The influence exerted by foreign proteins seems to be slight. To 0·06 g.

methaemoglobin was added 0·06 g. crystalline egg-albumin; the control lacked the egg-albumin. The solutions were acidified with $N/5$ HCl to p_H 3. The undenatured protein was estimated as alkaline methaemoglobin in the spectrocolorimeter. The amounts were 70 % in the experiment and 68 % in the control. A similar result was obtained for laked blood.

Salts have but a small effect on the recovery of undenatured protein. To varying concentrations of NaCl in a constant volume of aqueous solution (14 cc.) 1 cc. of 5 % methaemoglobin solution was run in. 5 cc. of $N/5$ HCl were added to the solution of salt and methaemoglobin, and after 10 minutes 5 cc. $N/5$ NaOH. Then the protein was treated with alkaline cyanide, ammonium sulphate ($\frac{1}{2}$ sat.), and estimated as HbCO. The results of two experiments are shown in Fig. 6.

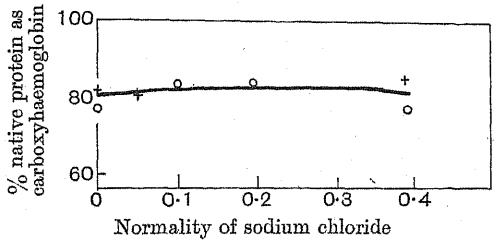


Fig. 6. Effect of increasing concentrations of NaCl on the yield of native protein.

The behaviour of the protein when subjected to repeated denaturation is of considerable interest. To protein in standard flasks was added HCl and after 5 minutes alkali in amount insufficient to neutralise the acid. For 5 cc. HCl were added 4·5 cc. $N/5$ NaOH. This was done once, twice, and three times. Then the protein was treated with cyanide, and estimated as HbCO. The yield of native material was never found to decrease on repetition of denaturation (Table IV).

Table IV.

No. of times acidified ...	1	2	3
	Percentage of native protein		
1	62·8	68	56
2	80	73	73
3	55	58	56

When the process is repeated but the protein made alkaline between each acidification, the yield of undenatured protein is still unaffected unless the denatured protein is separated from the native material, when the native residues denatured in the normal manner [Anson and Mirsky, 1929]. This observation has been confirmed by the author.

Discussion.

The experimental results described above present some remarkable features which it may be well to emphasise. The yields of "reversed" protein

seem largely independent of the temperature at which denaturation takes place; above a p_H of about 2·6 the extent of denaturation is completely independent of the p_H, and of the time of standing in acid solution. The steady state reached after a few minutes may be due to an equilibrium along the lines suggested by Anson and Mirsky, or to the whole of the protein being altered. It has been found that 100 % yield of native protein is never obtained, *i.e.* that some "irreversible" denaturation always takes place. (Wu and Lin state that they obtained complete "reversion" in one case.) It is very hard to conceive how the yield of "reversed" product can be independent of time and of repetition of acidification unless some alteration takes place in the whole of the protein. This change may be due to some denaturation in the classical sense of the word, followed by stabilisation of the remainder by the denatured product. If only part of the protein were denatured it might be expected that the residue of native material would become denatured as the protein stood in acid solution, and indeed the more so the longer it stood.

It is evident that there are two alternative views:

(*a*) the equilibrium view of Anson and Mirsky;

(*b*) the view that part of the protein is denatured and that this protects the remainder from further denaturation.

Now it has been shown that at acid p_H the spectrum of acid methaemoglobin can be detected, and, on reduction and aeration, that of oxyhaemoglobin. Some undenatured protein remains. Yet the amount of native material thus observed is always much smaller than that obtained by reversion. Some reversion may take place. But when the solution is made alkaline and the solution examined for alkaline methaemoglobin, the substance is seen in amount which accounts for the total native protein obtained, even though the whole protein is quantitatively precipitated on making isoelectric. Here we have direct evidence for the existence of some complex such as has been conceived in the second of the two suppositions. It may well be that in the acid solutions of protein studied the haematin group is entirely split off from the protein fraction, and that part of the free protein has become denatured, and has combined with the rest of the globin to form a complex stable to further treatment. On making alkaline it may be that the haematin fraction recombines with the globin and yields methaemoglobin, this being a rapid reaction. The alkali may further attack the complex between native and denatured protein, this being a slow reaction. Thus, if it be assumed that the complex between native and denatured material is insoluble at the isoelectric point, whether the protein is combined with haematin or not, we have a complete explanation of all the observed facts. For the alkaline methaemoglobin formed initially would thus be precipitated on neutralising at once; but after the slow dissociation had taken place between the native protein and the denatured, neutralisation would precipitate only the denatured fraction.

In the work here described the estimation of native protein falls ultimately

in every case on the formation of a conjugated protein. The validity of this procedure may be discussed with advantage. The question at issue is whether the formation of derivatives of globin, having spectra apparently identical with those of native protein, is a sufficient criterion for the existence of native globin. When a protein undergoes some secondary change, in every case it yields haemochromogen when coupled with reduced haematin [Anson and Mirsky, 1925]. Globin behaves as a typical protein in this respect. The action of acid, alkali, heat, light, shaking, urea, all give substances which have the haemochromogen spectrum. The least alteration in the protein appears to destroy its power of forming haemoglobin. This being the case it seems not improbable that were the protein concerned in these experiments altered in any fundamental way, the spectra of acid and alkaline methaemoglobin, of reduced and oxyhaemoglobin, and of carboxyhaemoglobin would not have been observed in solutions which could be entirely precipitated.

It has been suggested by Wu and Lin [1927] and by Anson and Mirsky [1929] that the sensitivity of oxyhaemoglobin to acids is greater than is that of reduced haemoglobin and they have suggested that the phenomena observed by Anson and Mirsky are due to the conjugation of the globin with the haematin. Anson and Mirsky have shown that similar effects may be obtained on acidifying solutions of globin, *i.e.* the prosthetic group may play some part in the phenomena, though not a decisive one. There is no evidence that like phenomena can be observed with other protein solutions.

Summary.

1. The formation of acid haematin from acid methaemoglobin has been studied as a function of p_H.

2. It has been found that some acid methaemoglobin appears to exist at p_H values at which the protein is entirely precipitable, and that hence reversion cannot account for all the native protein obtained after this procedure.

3. Curves have been obtained for the amount of soluble native protein obtained from methaemoglobin solutions brought to different p_H values.

4. On making an acid solution of protein neutral and then dissolving in the minimum of excess alkali, a solution is obtained which is entirely reprecipitated on neutralising with acid. If the solution be allowed to stand at an alkaline p_H the solubility increases, and finally a solution is obtained which is only partially precipitable on bringing to the isoelectric point.

5. A solution of protein made alkaline after having been acid shows the spectrum of alkaline methaemoglobin irrespective of whether the protein is soluble or insoluble at the isoelectric point, *i.e.* of the time through which the solution has stood alkaline. Estimation of the alkaline methaemoglobin in such solutions yields curves which coincide with those obtained after "reversion" by Anson and Mirsky's method.

6. Besides alkaline methaemoglobin, which precipitates entirely at the isoelectric point, solutions of reduced haemoglobin, oxyhaemoglobin, and

carboxyhaemoglobin can be made, which behave in a similar manner. The properties of all these solutions alter on standing, and the dissolved substances become only partially precipitable on neutralisation.

7. It is possible to obtain solutions at acid p_H which show spectra identical with that of carboxyhaemoglobin but which precipitate entirely on neutralisation. Estimation of the amount of native protein, measured by the intensity of this spectrum, yields curves similar to those obtained when the natural protein is estimated as alkaline methaemoglobin, or when it is subjected to the reversion procedure of Anson and Mirsky.

8. The methaemoglobin solution having been brought to a p_H of, say, 2·5, it has on no occasion been found possible to obtain all the material in the soluble native state, by any process whatever.

9. The author has confirmed the findings of Anson and Mirsky that the temperature at which denaturation takes place has little effect on the yield of native material after treatment with alkali, and that the extent of denaturation is unaffected by length of treatment with acid, after the first few minutes.

10. Denatured proteins combine with haematin to yield haemochromogen when the haematin is reduced, and parahaematin when it is oxidised. Yet the insoluble protein studied shows the spectrum of native derivatives of haemoglobin, reduced and oxy-haemoglobin, carboxyhaemoglobin, and alkaline methaemoglobin, in amount sufficient to account for all the native material obtained after treatment with alkaline cyanide. This phenomenon may be explained if it be assumed that there is formed a complex between the denatured fraction and the undenatured, this complex resembling denatured protein in being insoluble at the isoelectric point, and native protein in showing the typical spectrum of undenatured derivatives of haemoglobin.

I have much pleasure in expressing my thanks to Dr D. Keilin and Mr R. Hill for the help they have given me while this work was in progress, and to Sir F. G. Hopkins for the interest he has shown.

REFERENCES.

Anson and Mirsky (1925). *J. Physiol.* **60**, 50.
—— —— (1929). *J. Gen. Physiol.* **13**, 121.
—— —— (1930). *J. Gen. Physiol.* **13**, 469.
Arnold (1900). *Z. physiol. Chem.* **29**, 78.
Bertin-Sans and de Moitessier (1893). *Compt. Rend. Acad. Sci.* **114**, 923.
Chick and Martin (1910). *J. Physiol.* **40**, 404.
Cubin (1929). *Biochem. J.* **23**, 25.
Hartridge (1912). *J. Physiol.* **44**, 34.
Hill (1929). *Proc. Roy. Soc. Lond.* B **105**, 112.
Keilin (1926). *Proc. Roy. Soc. Lond.* B **100**, 129.
Lewis (1926). *Biochem. J.* **20**, 984.
Michaelis and Rona (1910). *Biochem. Z.* **29**, 494.
Spiegel-Adolf (1926). *Biochem. Z.* **170**, 126.
Stadie and Ross (1926). *J. Biol. Chem.* **68**, 229.
Van Klaveren (1901). *Z. physiol. Chem.* **33**, 293.
Wu (1929). *Chinese J. Physiol.* **3**, 1.
—— and Lin (1927). *Chinese J. Physiol.* **1**, 219.

CLXV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XX. ON THE PRODUCTION OF MANNITOL FROM HEXOSES AND PENTOSES BY A WHITE SPECIES OF *ASPERGILLUS*.

BY FREDERICK PHILIP COYNE AND HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received July 29th, 1931.*)

THE production of mannitol by bacterial fermentation has been extensively studied, since the organisms concerned are a source of trouble in the wine industry. Gayon and Dubourg [1901] showed that although glucose, sorbose, galactose, mannose, sucrose, maltose, lactose, raffinose and xylose are all fermented, laevulose alone yields mannitol. They found that arabinose and trehalose are not attacked. Peterson and Fred [1920, 1] reported that certain pentose-fermenting bacteria isolated from silage and manure also possess the power of producing mannitol. These organisms ferment glucose, galactose, mannose [Peterson and Fred, 1920, 2], xylose and arabinose [Fred, Peterson and Anderson, 1921], but again mannitol is obtained from laevulose alone, and not from any of the other sugars. With sucrose and raffinose some strains give traces of mannitol [Stiles, Peterson and Fred, 1925]. This fact was also observed by Gayon and Dubourg [1901], and it appears probable that fermentation of these sugars is not preceded by hydrolysis to monosaccharides, since invert sugar yields relatively large amounts of mannitol. It is most probable that mannitol arises by direct hydrogenation of one molecule of laevulose, coincidently with the oxidation of another molecule to lactic acid, acetic acid and carbon dioxide [Peterson and Fred, 1920, 1].

The presence of mannitol in the tissues of higher fungi was first discovered by Braconnot [1811], whose observation has been confirmed repeatedly and extended to the lower fungi. Obaton [1929], in a review on the evolution of mannitol in plants, studied in detail the production of mannitol and of trehalose in the mycelium of *Aspergillus niger*, but found no correlation between these two products, since factors affecting the one were without any effect on the other.

Recently Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that mannitol may be regarded as a product of mould fermentation. In the case of one white species of *Aspergillus* (Ac. 55), the yield of mannitol

approached 50 % of the glucose utilised. To obtain this result the rate of aeration must be carefully controlled. Since the source of mannitol is glucose, which on chemical reduction gives sorbitol, an intensive search was made for this alcohol, but no trace was discovered. The other metabolic products found in addition to mannitol were ethyl alcohol and carbon dioxide with traces of volatile and non-volatile acids.

In view of these results it was decided to extend this investigation, and the results of metabolism experiments on mannose, galactose, laevulose, xylose and arabinose are now reported. This work was restricted to one organism, *i.e.* that which gave the optimum yields of mannitol from glucose. In all experiments the medium consisted of 250 cc. of a 5 % solution of the sugar under investigation together with the usual Czapek-Dox salts, the medium being contained in a 750 cc. conical flask. It may be noted that this organism grows better in a medium where K_2HPO_4 is substituted for KH_2PO_4. The latter was used throughout, however, since the chance of a purely chemical decomposition of the sugar is thus decreased.

Experimental.

Carbohydrates.

A list of the sugars used in this work is given below, together with the observed optical rotations as a criterion of purity. The experiments with glucose, referred to in the introduction, were not repeated, but the results previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] have been incorporated in Table II for comparison.

d-*Mannose* (British Drug Houses), as obtained gave $[\alpha]^{22}_{5461} + 18{\cdot}1°$ and $[\alpha]^{22}_{5790} + 16{\cdot}1°$ in water (c, 0·9948).

d-*Xylose* (Swann Chemical Co., Birmingham, Alabama), was recrystallised from aqueous alcohol. The final product gave $[\alpha]^{18}_{5461} + 22{\cdot}05°$ and $[\alpha]^{18}_{5790} + 19{\cdot}12°$ in water (c, 0·2900).

d-*Galactose* (Pfanstiehl), as obtained gave $[\alpha]^{19}_{5461} + 93{\cdot}5°$ and $[\alpha]^{19}_{5790} + 83{\cdot}0°$ in water (c, 1·000).

l-*Arabinose* (British Drug Houses), as obtained gave $[\alpha]^{20}_{5461} + 120{\cdot}4°$ and $[\alpha]^{20}_{5790} + 106{\cdot}9°$ in water (c, 1·1341).

d-*Laevulose* (Merck), as obtained gave $[\alpha]^{22}_{5461} - 107°$ and $[\alpha]^{22}_{5790} - 94{\cdot}7°$ in water (c, 2·009).

Medium.

Sugar	25·00 g.
$NaNO_3$	1·00 g.
KH_2PO_4	0·50 g.
KCl	0·25 g.
$MgSO_4.7H_2O$	0·25 g.
$FeSO_4.7H_2O$	0·005 g.
Water	... to 500 cc.

The salts were dissolved separately and added to the solution of the sugar. 250 cc. of the medium were measured into each of two 750 cc. conical flasks and these were then sterilised by steaming for 30 minutes on each of three successive days.

Organism.

The organism used was an unnamed white species of *Aspergillus*, which was sent originally to Ardeer in 1923 by Dr Charles Thom, of the U.S. Department of Agriculture. It bore the Thom and Church Catalogue No. 4640·489, and the Ardeer Catalogue No. Ac. 55. Its carbon balance sheet was worked out [Birkinshaw, Charles, Raistrick and Stoyle, 1931] and it was then used for the preparation of mannitol from glucose [Birkinshaw, Charles, Hetherington and Raistrick, 1931]. All inoculations were made from beer-wort agar slopes grown at 25° for 5 days. A suspension in 5 cc. of distilled water of the heavily sporing mycelium from one test-tube culture was used for the inoculation of each flask of medium.

Incubation.

After inoculation, the two flasks were each fitted with a sterile rubber bung carrying an inlet glass tube which reached practically to the surface of the culture medium, and an outlet tube, the end of which only just passed through the bung. Each end of each tube was fitted with a cottonwool plug and the whole fitting—bung and glass tubes—was sterilised in the autoclave immediately before use. The inlet tube was connected to a controlled air supply and the outlet tube to a water seal, ensuring a closed system. The whole apparatus was maintained at a temperature of 25° (± 1°). Aeration was not commenced until a continuous felt of mycelium had been established (7–17 days). Thereafter 300 cc. of sterile air were passed into each flask daily, except Sundays. It had been shown previously that the omission of one day in seven only prolongs the period of incubation without affecting the ultimate result. After a few days of aeration, the thin film grew considerably and formed a thick white felt with a dry surface of sterile, woolly, aerial hyphae. This then remained unaltered, except for a slight wrinkling, until the supply of easily assimilable carbon became low, when the culture showed distinct signs of autolysis, shrinking and becoming water-logged. The flasks were then removed for examination.

With mannose, galactose and xylose the growth appeared normal and similar in character to that obtained on glucose. With arabinose, however, even after 3 months no sign of autolysis had appeared. One flask (*a*) was therefore removed after 93 days and examined. The other flask (*b*) was incubated for 11 more days and then, as autolysis became evident, it was also removed for examination. The residual sugar in both was high and, as will be seen later, the yield of mannitol was very much less than with glucose, mannose, galactose and xylose.

With laevulose the results were surprising, as after 3 weeks of incubation only rudimentary growth had occurred. A few tiny islets of mycelium formed very slowly, but even after 5 weeks these showed no signs of further development. It was thought possible that the laevulose might contain a trace of some toxic substance. That this was not so was shown by the abundant growth of other moulds on the same medium. Samples from one of the flasks were transferred to test-tubes, sterilised by one steaming for 30 minutes, and inoculated with six different species of *Aspergillus* and *Penicillium*. All of these showed normal growth in a few days.

A second attempt was made to grow the white *Aspergillus* on a fresh batch of the same medium. In spite of very heavy inoculations the same result was obtained—traces of growth which showed no appreciable development after the first fortnight. Since this organism prefers an alkaline medium, 2·0 cc. of sterile *N* NaOH were added to each flask after 3 weeks. This is equivalent to converting the acid phosphate into the dibasic salt. The flasks were shaken thoroughly to ensure mixing, but were not inoculated again since definite traces of growth were present. Ten days later a distinct improvement was apparent. In both flasks a ring of growth could be observed round the edge of the surface, together with a few discrete colonies. Aeration was now commenced, but the growth remained almost stationary, and 9 weeks later the flasks were removed for analysis.

Examination of metabolism solution.

The following procedure was adopted in all the experiments. The metabolism solution was filtered, without sterilisation, from the mycelium, which was washed 5 times with boiling water. Filtrate and combined washings from two flasks, corresponding to 500 cc. of original medium, were made up to 1 litre, constituting solution A. Aliquot portions of this were used for the various analyses and for the isolation of mannitol.

The collected results are given in Table II in which the results for glucose previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] are incorporated.

The following estimations were carried out.

I. *Residual sugar*, (*a*) by polarimeter and (*b*) by Shaffer and Hartmann method.

(*a*) By polarimeter. The solution was polarised in a 4 dm. tube using both the mercury green and yellow lines, $\lambda 5461$ and $\lambda 5790$.

(*b*) By the Shaffer and Hartmann method. The normal procedure was followed without modification, the factors for the different sugars being determined by control estimations carried out on standard solutions of each sugar.

The results obtained by the two methods are given in item 4, Table II, and are in good agreement throughout.

II. *Acidity produced.* This was determined by titration with standard sodium hydroxide to phenolphthalein. The figures given in item 5, Table II,

Table I.

			Mannose	Galactose	Laevulose	Xylose	Arabinose (*a*)	Arabinose (*b*)
1	Concentration of sugar in standard metabolism solution A, calculated from mean polarimeter results (g. per 100 cc.)		Nil	0·516	1·46	0·290	0·735	0·750
2	Volume in cc. of metabolism solution A made up to 50 cc. with 3 g. of borax (solution B)		40·0	40·0	40·0	25·0	40·0	40·0
3	Concentration of sugar in solution B (g. per 100 cc)		Nil	0·413	1·168	0·145	0·588	0·600
4	Rotation of solution B in 4 dm. tube	λ 5461	+1·17°	+0·38°	−1·39°	+0·50°	+0·67°	+0·42°
		λ 5790		+0·36°	−1·27°	+0·45°	+0·57°	+0·39°
5	Rotation in 6·0 % borax of synthetic sugar solution of same concentration as in solution B	λ 5461	Nil	−0·59°	−1·39°	+0·04°	+0·29°	+0·29°
		λ 5790		−0·52°	−1·27°	+0·04°	+0·26°	+0·26°
6	Rotation due to mannitol (Item 4–Item 5)	λ 5461	+1·17°	+0·97°	Nil	+0·46°	+0·38°	+0·13°
		λ 5790		+0·88°	Nil	+0·41°	+0·31°	+0·13°
7	Percentage mannitol in solution B. Calculated from Item 6	λ 5461	0·730	0·60	Nil	0·27	0·22	0·084
		λ 5790		0·61	Nil	0·27	0·20	0·074
8	Total mannitol in metabolism solution A (g.)	λ 5461	9·13	7·5	Nil	5·4	1·4	0·53
		λ 5790		7·6	Nil	5·4	1·3	0·46

Table II.

		Glucose	Mannose	Galactose	Laevulose	Xylose	Arabinose (*a*)	Arabinose (*b*)
1	Incubation period (days)	77	65	72	94*	70	93	104
2	Aeration period (days)	70	49	55	62	60	83	96
3	Initial sugar (g.)	25·0	25·0	25·0	25·0	25·0	12·5	12·5
4	Residual sugar (g.) (*a*) by pol. λ 5461	—	Nil	5·13	14·6	2·90	3·66	3·75
	λ 5790	—	Nil	5·18	14·6	2·90	3·70	3·75
	(*b*) by Shaffer-Hartmann	0·34	Nil	4·55	14·6	—	3·57	4·15
					Decrease of		Decrease of	Decrease of
5	Acidity produced (cc. *N*/NaOH)	1·0	0·2	0·08	0·3	0·4	0·8	0·9
6	Mannitol estimated by polarimeter (g.)	8·4	9·1	7·5	Nil	5·4	1·35	0·50
7	Mannitol isolated (g.)	6·9	9·3	7·4	Nil	4·9	0·2†	0·1†
8	Percentage estimated yield of mannitol on sugar utilised	34·2	36·4	37·8	Nil	24·4	15·3	5·7
9	Percentage yield of mannitol isolated on sugar utilised	28·0	37·2	37·3	Nil	22·2	—	—

* NaOH (2 cc. *N*) added after 22 days' incubation.

† Isolated as tribenzylidene compound.

have been corrected for the original acidity of the medium. The acidity produced was practically negligible in all cases.

III. *Mannitol estimated by polarimeter.* The method used was that described by Raistrick and Young [1931] for the estimation of mannitol in fermentation solutions. This method is based on the fact that, although in aqueous solution mannitol gives a very small rotation, $[\alpha]^{20}_{5461} - 0{\cdot}49°$, in presence of 6·00 % borax a high positive rotation is given, the exact values of which vary with the concentration of mannitol and are given by the above authors. Since, however, the optical rotations of all the sugars used are greatly affected by the borax, a correction must be made for this effect. The procedure was as follows. The concentration of sugar in the metabolism solution A having been determined as described in Section I, a measured volume of the metabolism solution, together with 3·00 g. of borax ($Na_2B_4O_7 . 10H_2O$) was made up to a volume of 50 cc. and polarised. A solution of the sugar in question, in the same concentration as above, was also polarised in presence of 6·00 % borax. The difference between these two readings gave the rotation due to mannitol. The results obtained are given in Table I.

Isolation of mannitol.

A measured volume of the metabolism solution having been retained for the above estimations the remainder was used for the isolation of the product. 10 cc. of basic lead acetate solution were added and the precipitate was allowed to settle overnight. Excess lead was removed from the filtrate by H_2S and the solution was then concentrated *in vacuo* to 20–50 cc., the temperature of the water-bath being kept below 50°. At this stage a certain amount of crystalline material had usually separated. It was dissolved by warming and then 2 volumes of boiling absolute alcohol were added. On cooling, a voluminous crystalline mass of fine white needles was obtained. This was filtered, washed, dried and weighed. The mother-liquors were concentrated again and the process repeated until no further crystalline product separated.

The residual sugar remained in solution and did not interfere with the crystallisation of the mannitol, except in the case of arabinose. As shown in Table II, the two flasks of arabinose were examined separately, each solution being made up to a volume of 500 cc. No mannitol was isolated from either by direct crystallisation. With the first one a yield of 2·5 g. of white crystalline material was obtained, and estimations of reducing power and rotation showed that this product contained about 80 % of arabinose. The small amount of mannitol present could not be separated by direct means. It was, therefore, converted into the tribenzylidene derivative [Meunier, 1891] which was readily isolated and purified, as the sugar does not yield a benzylidene compound with HCl and benzaldehyde. The method, however, is not quantitative, only about 60 % of the calculated yield being obtained from synthetic mixtures of arabinose and mannitol. With the second arabinose metabolism solution no attempt was made to isolate a crystalline product directly. The concentrated

solution was desiccated *in vacuo* over P_2O_5 and then treated with HCl and benzaldehyde, by which means tribenzylidene mannitol was again obtained.

Each fraction of crystalline material obtained by the above treatment from the mannose, galactose and xylose metabolism solutions had M.P. between 158° and 163°. On recrystallisation from aqueous alcohol all fractions had M.P. 165–166° which was not depressed on mixing with an authentic specimen of pure mannitol (M.P. 166°). The optical rotation was also measured in each case and found to be $[\alpha]^{20}_{5461} - 0{\cdot}49°$ in water.

From each of the purified samples the tribenzylidene compound was prepared by the action of concentrated HCl and benzaldehyde [Meunier, 1891]. After recrystallisation from absolute alcohol the M.P. was 217–219°. The mixed M.P. with tribenzylidene mannitol was 217–219°. The optical rotation found was $[\alpha]^{17}_{5461} - 17{\cdot}3°$ and $[\alpha]^{17}_{5790} - 15{\cdot}6°$ in chloroform; Patterson and Todd [1929] give $[\alpha]^{25}_{5461} - 17{\cdot}5°$ and $[\alpha]^{25}_{5790} - 15{\cdot}5°$.

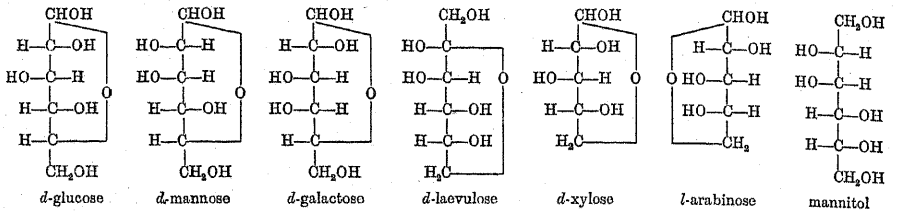
As a final confirmation of the identity of the mannitol a weighed amount of each specimen was polarised in 6·00 % borax solution when the calculated results agreed with the actual amounts used.

In the case of arabinose, as already stated, the mannitol was isolated as the tribenzylidene derivative. With laevulose no mannitol could be detected by the polarimetric method and none was isolated.

Discussion.

As is shown in Table II, the yield of mannitol from glucose, mannose and galactose is approximately the same, about 35 %. Xylose produced 24 %, but it is doubtful whether one can place any significance on this ratio, since Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that with unrestricted aeration the organism slowly utilises the mannitol formed. With arabinose the growth is less satisfactory and the yield of mannitol, although still appreciable, is poorer. Laevulose is apparently not a satisfactory source of carbon for this organism and is metabolised very slowly. The residual metabolism solution, however, contained only 60 % of the original sugar, so that 10 g. of laevulose had been utilised. In spite of this, no mannitol could be isolated and the rotation of the solution in 6·00 % borax confirmed the absence of even traces of this product (Table I). It may be that, since laevulose is so resistant to attack, any mannitol formed is broken down preferentially, but even were this the case, one would expect to find a small amount in the solution.

These results are rather unexpected, running as they do exactly contrary to the results of bacterial fermentation. In the latter case mannitol is produced from laevulose and not from any other hexose or pentose, while with this species of *Aspergillus* mannitol is obtained from all the sugars so far tested with the exception of laevulose. A consideration of the structural formulae renders the problem even more complex.



Mannose would naturally yield mannitol by direct hydrogenation, and for glucose one could postulate a Lobry de Bruyn type of transformation. Acceptance of such a hypothesis is rendered difficult by the laevulose results—since this sugar also gives mannose by the Lobry de Bruyn transformation, especially in alkaline solution—and becomes untenable when the pentoses are considered.

It might be claimed that the theory advanced to account for the alcoholic fermentation of sugar by yeast can be applied. Both the yeasts and the bacteria, however, show a fundamental difference in their metabolism as compared with the fungi. In the first cases the process is almost exclusively "catabolic" while with the fungi syntheses of an "anabolic" type are a striking characteristic. We have no evidence at present as to whether a hypothetical intermediate product, of the type of methylglyoxal, *etc.*, is transformed directly into mannitol. It appears at least equally possible that a hypothetical intermediate product is first built up into a reserve polysaccharide which is broken down as required and forms the immediate precursor of mannitol, which latter must then be regarded as a product of indirect synthesis. It is proposed to investigate this point by examination of the mycelium at various stages of growth, the mannitol produced being estimated at the same time.

Summary.

The metabolism of a species of white *Aspergillus* has been studied on synthetic media with controlled aeration. Mannitol is formed by this organism in considerable amounts (15–35 % of utilised sugar) from glucose, mannose, galactose, xylose and arabinose. The organism does not, however, produce mannitol from laevulose. These results are directly contrary to those reported for mannitol-producing bacteria.

This work has been rendered possible by a grant to one of us (F.P.C.) from the Research Council of Imperial Chemical Industries, Ltd., to whom we tender our best thanks.

REFERENCES.

Birkinshaw, Charles, Hetherington and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 153.
Birkinshaw, Charles, Raistrick and Stoyle (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 42.
Braconnot (1811). *Ann. Chim.* (1), **79**, 265.
Fred, Peterson and Anderson (1921). *J. Biol. Chem.* **48**, 385.
Gayon and Dubourg (1901). *Ann. Inst. Pasteur*, **15**, 527.
Meunier (1891). *Ann. Chim.* (6), **22**, 420.
Obaton (1929). *Revue Gén. Bot.* **41**, 282, 365, 424, 498, 555, 622.
Patterson and Todd (1929). *J. Chem. Soc.* 2876.
Peterson and Fred (1920, 1). *J. Biol. Chem.* **41**, 431.
—— —— (1920, 2). *J. Biol. Chem.* **42**, 272.
Raistrick and Young (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 173.
Stiles, Peterson and Fred (1925). *J. Biol. Chem.* **64**, 643.

CLXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXI. QUANTITATIVE EXAMINATION BY THE CARBON BALANCE SHEET METHOD OF THE TYPES OF PRODUCTS FORMED FROM GLUCOSE BY SPECIES OF BACTERIA.

By JOHN HOWARD BIRKINSHAW,
JOHN HENRY VICTOR CHARLES
and PERCIVAL WALTER CLUTTERBUCK.

From the Biochemical Department, Ardeer Factory (Imperial Chemical Industries, Ltd.), Stevenston, Ayrshire and the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received July 29th, 1931.*)

In an earlier paper of this series [Birkinshaw and Raistrick, 1931], a full description was given of a method for the determination of the products formed by micro-organisms by the carbon balance sheet method, with particular reference to moulds. A number of balance sheets in later papers [Raistrick *et al.*, 1931] show that by this means a useful general survey of the types of mould metabolism products can be obtained and may be used as a basis for the biochemical classification of these organisms. The method as originally evolved, however, was intended to cover the whole field of micro-organisms, and the present work was undertaken largely with a view to determining how far the original method would apply, and what modification would be necessary, in order to study the metabolic products of bacteria. In addition it was hoped to confirm and supplement in a quantitative manner the results obtained by Harden, Neuberg, Kluyver and other workers in this field. It was further anticipated that some of the organisms examined would show hitherto unsuspected amounts of some types of products, or of "carbon unaccounted for," and would thus open up interesting possibilities in the way of detailed investigation of the metabolism of the organism so selected.

Medium.

The Czapek-Dox-glucose medium used in the work on moulds was purposely chosen on account of its simplicity and its lack of any source of carbon other than glucose. Moulds are remarkably tolerant of acid, consequently the low p_H obtained with this medium when organic acids are produced does not markedly affect the growth or activity of these organisms. With bacteria, the

case is otherwise. They are known to be much more sensitive to acidity, and it was necessary, therefore, to adopt some method of neutralising the acid as formed. Both phosphate buffering and addition of calcium carbonate were tried, the latter proving more useful. Growth of *B. coli* (used as a test organism) on Czapek-Dox medium modified by addition of carbonate was unsatisfactory. It was considered that the form of nitrogen ($NaNO_3$) of the medium was probably unsuitable and a series of the commoner bacteria was therefore sown on to a Czapek-Dox medium containing the nitrogen in various forms, but in each case equivalent in nitrogen to the 0·2 % sodium nitrate of the original medium. Only 1 % of glucose was used so that an accumulation of acid was impossible, and calcium carbonate could thus be omitted without detriment to growth. The growth of the organisms after 5 days on the modified medium is indicated in Table I.

Table I. *Growth of stock strains of bacteria on modified Czapek-Dox solution containing various sources of nitrogen. Temp. 34°.*

		Time of incubation: 5 days								48 hours
		Sod. nitrate	Sod. nitrite	Micro-cosmic salt	Amm. nitrate	Amm. sulphate	Amm. chloride	Calc. nitrate	Pot. nitrate	Micro-cosmic salt + $CaCO_3$
C 1.	*B. mesentericus*	+++	−	+++	−	+++	+++	−	−	+++
C 3.	*B. coli* (*Escherichia coli*)	−	−	+++	++	++	++	−	−	+++
C 4.	*B. proteus-vulgaris*	−	−	−	−	−	−	−	−	·
C 6.	*Bact. acidi-lactici* (*Escherichia acidi-lactici*)	−	−	+++	++	+	++	−	−	+++
C 12.	*Aerobacter cloacae*	−	−	+++	+	+++	+++	−	−	+++
C 13.	*B. figurans*	−	−	−	−	−	−	−	−	·
C 17.	*Serratia marcescens*	−	−	−	−	−	−	−		·
C 24.	*B. lactis-aerogenes* (*Aerobacter aerogenes*)	+++	−	+++	++	+++	·	−		++++
C 25.	Leather bac. Houston	++	−	+++	+	−	++	−	+++	++++
C 26.	Lactose acidifier (*Aerobacter*)	−	−	++	−	−	−	−	−	+
C 27.	Lactose fermenter (*Aerobacter*)	++	−	+++	+	+++	+++	−	+++	+++
C 28.	*B. mycoides*	−	−	+++	+++	+++	+++	−	+++	++
C 30.	Sporing rod bacillus	++	−	+++	+++	+	++	−	+++	++
C 33.	*Sarcina lutea*	−	−	−	−	−	−	−	−	·
C 34.	*B. coli* (*Escherichia coli*)	−	−	+++	+	+	++	−	−	+++

The medium contained only 1 % of glucose.

The microcosmic salt ($NaNH_4HPO_4.4H_2O$) medium showed definitely the best growth for the largest number of bacteria and was employed in a further trial. This time 1 % of calcium carbonate was added to the medium to determine whether this inhibited growth, since it would be necessary or at least desirable to use 5 % of glucose in the metabolism experiments with a correspondingly greater acid production. No inhibition was observed. The growth is shown in the last column of Table I.

It was thus evident that a number of organisms could be selected which grew rapidly on a medium containing merely inorganic salts apart from the glucose. A further batch of cultures shown in Table II yielded in most cases disappointing results, in respect of their growth on this medium.

Table II. *Growth of various stock strains of bacteria on modified Czapek-Dox solution containing various sources of nitrogen.*

Time of incubation 3 days at 34°.

	Sod. nitrate	Sod. nitrite	Amm. chloride	Amm. sulphate	Pot. nitrate	Calc. nitrate	Micro-cosmic salt	Amm. nitrate
C 38. *Streptococcus lacticus*	−	+	−	+	−	−	−	−
C 39. *B. guntheri* (*Streptococcus lactis*)	−	?	−	++	−	−	+	−
C 40. *B. bulgaricus* (*Lacto-bacillus bulgaricus*)	+	+	−	−	+	−	−	+
C 41. *B. acidophilus* (*Lacto-bacillus acidophilus*)	?	−	−	−	−	−	−	−
C 44. *B. pyocyaneus* (*Pseudomonas aeruginosa*)	++	−	++	+++	+++	−	+++	+
C 46. *Pseudomonas* (denitrifying)	+	−	−	−	−	−	+++	−
C 54. *B. acidi-lactici*	−	−	+	+	−	−	++	+
C 60. *B. faecalis-alcaligenes*	−	−	−	+	−	−	+	−
C 62. *B. acidi-lactici* (St Georges)	−	−	+	+	−	−	++	+
C 66. *B. butyricus* (Hueppe)	−	−	−	−	−	−	−	−
C 67. *B. mesentericus*	−	−	−	−	−	−	−	−
C 70. *B. asiaticus-mobilis*	++	−	+++	++	+	+	++++	+
C 73. *B. alkalescens*	−	−	+	−	.	−	++	−
C 80. *Mycobact. phlei*	−	−	−	−	.	−	−	−
C 81. Butter bac. (Rabinowitsch)	−	−	+	−	.	−	+	−
C 82. Moeller's "Mist" bacillus	−	−	++	+	.	−	++	+

At this stage it became evident that the number of species which would grow on a purely synthetic medium, containing only inorganic salts and glucose, was disappointingly small, and it appeared possible that the addition of amounts of organic nitrogen which would introduce carbon in quantities representing only a small percentage of that present as glucose would render a much larger number of bacteria available, whilst not appreciably affecting the carbon balance. Large amounts of organic nitrogen would be out of the question, since they would vitiate the carbon balance or, at least, necessitate a cumbersome technique. Growth on media obtained by addition of 2 drops of 10 % peptone, 10 % asparagine, caseinogen digest or double strength yeast water to 10 cc. of the Czapek-Dox microcosmic salt medium was compared for a series of organisms, which failed to grow in the absence of organic nitrogen (see Table III).

Table III. *Effect of additions of small amounts of organic nitrogen to modified Czapek-Dox medium.*

Incubated 4 days at 34°.

	Peptone	Asparagine	Caseinogen digest	Yeast water
C 38. *Streptococcus lacticus*	+	−	+	−
C 39. *B. guntheri* (*Streptococcus lactis*)	++	−	−	+
C 40. *B. bulgaricus* (*Lacto-bacillus bulgaricus*)	+	−	−	−
C 41. *B. acidophilus* (*Lacto-bacillus acidophilus*)	−	−	−	−
C 60. *B. faecalis-alcaligenes* (*Alcaligenes faecalis*)	+	−	++	−
C 66. *B. butyricus* (Hueppe)	++	−	++	−
C 67. *B. mesentericus*, No. 4	+	−	+++	++
C 80. *M. phlei*	+	−	+	+++
C 81. Butter bac.	++	++	++	++

As the addition of peptone appeared to give the best results it was adopted as a standard constituent to be added to the medium. The amount was fixed at 0·2 % corresponding to only 4 % by weight of the sugar, since this produced a considerable increase in growth whilst not interfering appreciably with the carbon balance sheets.

With the series of bacteria growing best at 25–30° the inorganic nitrogen proved relatively inefficient though here again the microcosmic salt was most favourable. The addition of peptone again rendered the medium much more suitable for bacterial growth.

The Czapek-Dox medium had been rather arbitrarily selected as the synthetic medium owing to its previous use for mould metabolism experiments. In order to justify its retention it was compared with other synthetic media, notably those of Prazmovski and Raulin (the latter *minus* tartrate). No advantage was obtained by the use of these two media, in fact the Czapek-Dox microcosmic salt and peptone medium was definitely superior.

The modified Czapek-Dox media employed were then as follows.

Medium A.

Microcosmic salt ($NaNH_4HPO_4$, $4H_2O$)	4·921 g.
K_2HPO_4	1·00 g.
KCl	0·50 g.
$MgSO_4$, $7H_2O$	0·50 g.
$FeSO_4$, $7H_2O$	0·01 g.
Glucose	50·00 g.
$CaCO_3$	28·00 g.
Distilled water	1000 cc.

250 cc. of medium, in a 750 cc. conical flask, were used in each experiment.

Medium B. To the above medium was added 0·2 % of peptone for certain species in order to increase the range of species which could be employed.

Medium C. This medium represented an attempt to eliminate the use of calcium carbonate by the employment of a phosphate solution of high buffering power. Medium C contains 8·70 g. of dipotassium phosphate per litre in place of 1 g. as in Medium A, the calcium carbonate being omitted. Thus glucose represents the only source of carbon, resulting in simplification of the balance sheet. Only two examples of its use are given.

Modifications in technique.

Certain departures from the standard technique as evolved for the study of moulds were necessitated by the different habits of growth of bacteria and by the difference in the medium used.

When calcium carbonate was employed, a known amount was sterilised dry in a plugged tube which was weighed, and the contents were added to the metabolism flask under aseptic conditions. The empty tube and plug were next weighed and the exact weight of carbonate obtained by difference. The flask was then sown with the organism to be examined and incubated in an apparatus similar to that used in the mould work.

In order to allow for a considerable evolution of carbon dioxide from the action of organic acids on the calcium carbonate present, a new type of potassium hydroxide absorption bulb was fitted immediately after the first

sulphuric acid bubbler. In this the carbon dioxide entering was allowed to impinge on the surface of the potassium hydroxide solution, the delivery tube being cut off about half an inch above the surface of the absorbent liquid. Much of the carbon dioxide was removed in this manner without any danger of blocking the tube with solid potassium bicarbonate, as occasionally happened with the moulds.

The air supplied to aerobic bacteria was the same as for moulds, *viz.* about 500 cc. daily. The air inlet tube in most cases dipped below the surface of the medium, so that the air on entering bubbled through the liquid and to some extent agitated the calcium carbonate. If the bacteria under examination were known to be film formers, however, the standard mould procedure was adopted, the air being admitted above the liquid surface. In the case of anaerobic bacteria the air was swept out of the apparatus with a current of nitrogen, and no further attention was given until the end of the experiment when the gaseous metabolism products were again removed by nitrogen.

After the incubation had proceeded for the length of time necessary to metabolise the greater part of the sugar as determined from a control experiment the apparatus was swept out with a current of air (or nitrogen) and then the several portions of the apparatus treated substantially as in the mould experiments. Only in the case of the flask contents was a modification necessary.

No preliminary titration of the flask contents was undertaken owing to the presence of calcium carbonate. An idea of the amount of acid formed is obtained from the amount of carbonate which disappears during the experiment. This approximate figure is inserted in the balance sheet in place of the figure obtained from the titration of the metabolism solution.

The contents of the flask are filtered through a filter-paper on which is distributed a weighed amount (about 0·5 g.) of "Supercel Hyflo" in place of kieselguhr. This was found to give easier filtration and to be practically carbon-free. A further weighed amount (1·5 g.) was added to the metabolism solution which was well shaken before filtration. The dried bacterial or other insoluble residue, together with the unused calcium carbonate and the supercel, remains on the paper. After drying it peels off readily and is weighed and ground up in a mortar. The residual carbonate present is determined by warming a portion of the mixture with dilute acid in the wet combustion apparatus, the carbon dioxide being determined in the usual way. On another portion a total carbon estimation is performed. In this way the value for "carbon in bacteria" is obtained.

The main filtered solution is made up to a known volume (500 cc.) and the determinations necessary to construct a carbon balance sheet are made.

It was found that lactic acid and butylene glycol, which would normally be included in the "carbon unaccounted for" are such frequent products of bacterial metabolism that the separate determination of them was necessary, in order to decide whether other products were present in this fraction.

Procedure.

The residue from the determination of the "volatile neutral carbon" (for which 100 cc. is taken in the ordinary method) is made up to 100 cc. and 25 cc. removed. This neutral solution is extracted with ether for some 12 hours in a continuous apparatus. This has the effect of removing all the butylene glycol (along with any compounds of similar nature, if present). The extract is set aside for glycol determination and the residue is acidified with phosphoric acid and the extraction continued for another 12 hours. The resulting extract is used for the estimation of lactic acid.

Determination of butylene glycol.

Preliminary tests of the method suggested by Malaprade [1928] for polyhydric alcohols showed that butylene glycol when oxidised by periodic acid behaves in a similar manner to ethylene glycol. One molecule of butylene glycol requires 1 atom of periodate oxygen according to the equation:

$$CH_3.CHOH.CHOH.CH_3 + HIO_4 = 2CH_3CHO + HIO_3 + H_2O.$$

Although this method would not in itself distinguish between butylene glycol and other polyhydric alcohols, it is a valuable aid to the construction of the carbon balance sheets when applied with discrimination and supplemented in doubtful cases by specific tests for, or by the isolation of, butylene glycol.

A standard solution of potassium periodate was made up such that on acidification and addition of excess potassium iodide it yielded a $N/10$ solution of iodine (calculated on the original volume). A known amount of the material extracted by ether from the neutral metabolism solution containing not more than 0·05 g. of butylene glycol was treated with 100 cc. of the periodate solution and 10 cc. of 10 % sulphuric acid. The mixture was allowed to stand overnight and then an excess of potassium iodide added and the free iodine titrated with $N/10$ thiosulphate. This titration figure must be more than 75 cc. which would correspond with the total conversion of the reagent.

Determination of lactic acid.

The method involving distillation with dilute permanganate and titration of the acetaldehyde evolved has been frequently modified, though the original form of the method is associated with the names of von Fürth and Charnass [1910].

The actual procedure is as follows.

A known amount of the solution containing 0·5–5·0 mg. of lactic acid is introduced into a Claisen flask together with 10 cc. of 10 % sulphuric acid. The flask is attached to an efficient Liebig condenser and a receiver consisting of a Volhard flask containing 20 cc. of $N/20$ sodium bisulphite. The Claisen flask is heated in a boiling water-bath and a current of steam passed in through

one neck. Through the other neck $N/200$ potassium permanganate solution is slowly dropped in until the liquid in the flask attains a permanent pink colour.

After a further 5 minutes the steam is shut off, the condenser washed down into the bisulphite and the contents of the Volhard flask washed out into a conical flask. Starch is added and the excess bisulphite is titrated with $N/20$ and finally $N/1000$ iodine until a pale blue colour is obtained. Excess of saturated sodium bicarbonate solution is then added to decompose the aldehyde-bisulphite compound and the solution is titrated with $N/1000$ iodine to re-establish the colour. With 1 cc. of 0·0997 % lithium lactate duplicate titrations obtained were 20·2 and 20·9 cc. of $N/1000$ iodine. The mean 20·55 cc. corresponds to 0·986 mg. lithium lactate, giving an error of 1 %.

The one difficulty which remains is due to the slight volatility of lactic acid. During the distillation of the volatile acids a certain amount of the lactic acid present distils over and is recorded as volatile acids. Since a separate lactic acid estimation is performed on another portion of the metabolism solution the carbon accounted for is slightly higher than it should be. This is borne out by the fact that in some cases the total carbon accounted for is higher than that present in solution. The error introduced in this manner is however fairly small. In distillation tests carried out with lactic acid present in about double the amount that was present in any metabolism experiment, lactic acid distilling over under the conditions of the experiment corresponded to 0·4 g. of carbon. This would, of course, represent a maximum error; it will be seen from the balance sheets that the actual error is usually considerably less, since the total volatile acid is frequently less than this figure even when lactic acid is present.

An attempt was made to calculate the respiration coefficient as for the moulds but when calcium carbonate is present there is difficulty in distinguishing between carbon dioxide arising from the carbonate and that due to respiration. It was assumed, however, for this purpose that all the carbonate disappearing had given rise to the theoretical amount of carbon dioxide, which was therefore deducted from the total carbon dioxide recovered.

Since the weight of bacteria was only determinable indirectly and was in any case small, this weight could not be accurately obtained. Hence the figure for the % carbon in bacteria was unreliable and was not determined although, of course, the total carbon in the bacteria was estimated.

It was found that a number of the organisms which grow quite well on the medium selected are unable to metabolise more than a small portion of the sugar supplied. Consequently, the standard procedure adopted was to carry out a control experiment with each organism giving good growth on the synthetic medium.

In these control experiments (Table IV) the organism was sown into a flask, fitted with tubes for aeration, and containing the medium as used in the metabolism experiments. It was incubated at a suitable temperature,

Table IV.

Cat. No.	Name	Source	Medium	Temp. °C.	Days	Sugar % Start	Sugar % Finish
C 39.	*B. güntheri* (*Streptococcus lactis*)	N.C.T.C. No. 661	B	34	26	5·02	3·16
C 38.	*Streptococcus lacticus*	N.C.T.C. No. 1551	B	34	21	5·17	3·74
C 87.	*Leuconostoc* species	Isolated from molasses at Ardeer	B	34	16	5·02	1·83
C 79.	*M. flavus* (Flugge)	N.C.T.C. No. 1629	B	28	14	5·39	5·25
C 78.	*M. roseus* (Bumm)	N.C.T.C. No. 195	B	28	28	4·91	4·63
C 44.	*B. pyocyaneus* (*Pseudomonas aeruginosa*)	N.C.T.C. No. 1999	A	34	8	5·14	4·68
C 44.	*B. pyocyaneus* (*Pseudomonas aeruginosa*)	N.C.T.C. No. 1999	B	34	31	5·02	2·44
C 46.	*Pseudomonas* (dentrifying)	N.C.T.C. No. 933	A	34	6	5·14	4·56
C 35.	*B. rossicus* Kell. (*Cellulomonas rossica*)	N.C.T.C. No. 1655	A	34	11	5·14	4·83
C 4.	*B. proteus-vulgaris* (*Proteus vulgaris*)	N.C.T.C. No. 67	B	34	6	5·21	3·66
C 47.	*B. phytophthorus* Appel	N.C.T.C. No. 1985. Dr E. F. Smith	B	28	20	5·50	3·12
C 45.	*B. aroideae* Townsend (*Erwinia aroidea*)	N.C.T.C. No. 1987. Dr E. F. Smith	B	28	31	5·35	0·10
C 51.	*B. carotovorus* Jones (*Erwinia carotovora*)	N.C.T.C. No. 1975. Dr E. F. Smith	B	28	27	4·56	1·90
C 43.	*B. marginale* N. A. Brown (*Phytomonas marginale*)	N.C.T.C. No. 1981. Dr E. F. Smith	B	28	15	5·12	4·70
C 50.	*B. campestre* Pammel (*Phytomonas campestre*)	N.C.T.C. No. 1958. Dr F. E. Smith	B	28	17	5·45	4·60
C 63.	*B. hyacinthi* Wakker	N.C.T.C. No. 387	B	28	26	5·54	4·68
C 64.	*B. aptatum* Brown and Jamieson (*Phytomonas aptata*)	N.C.T.C. No. 1977. Dr E. F. Smith	B	28	28	4·89	2·41
C 65.	*B. malvacearum* E. F. Smith (*Phytomonas malvacera*)	N.C.T.C. No. 1960. Dr E. F. Smith	B	28	11	5·31	5·20
C 75.	*B. cannae* Bryan (*Phytomonas cannae*)	N.C.T.C. No. 1956. Dr E. F. Smith	B	28	15	5·49	5·48
C 54.	*B. acidi-lactici*	N.C.T.C. No. 87	B	34	6	5·02	2·24
C 6.	*B. acidi-lactici* Grotenfelt, Hueppe (*Escherichia acidi-lactici*)	N.C.T.C. No. 413	A	34	6*	4·91	2·19
C 62.	*B. acidi-lactici* (St Georges)	N.C.T.C. No. 123	B	34	10	5·02	0·36
C 34.	*B. coli* Esch. (*Escherichia coli*)	N.C.T.C. No. 86	A	34	6*	4·91	2·51
C 37.	*Esch.* species	Isolated from milk at Ardeer	A	34	6*	4·91	1·09
C 26.	*Esch.* species	Isolated from milk at Ardeer	A	34	6*	4·91	3·12
C 24.	*B. lactis-aerogenes* Esch. (*Aerobacter aerogenes*)	N.C.T.C. No. 418	A	34	9*	4·91	0·02
C 24.	*B. lactis-aerogenes* Esch. (*Aerobacter aerogenes*)	N.C.T.C. No. 418	C	34	7*	4·93	1·37
C 12.	*Aerobacter cloacae*	Isolated at Ardeer	A	34	6*	4·91	0·86
C 12.	*Aerobacter cloacae*	Isolated at Ardeer	C	34	6*	4·93	2·77
C 25.	Leather bacillus (Houston)	N.C.T.C. No. 672	A	34	6*	4·91	0·08
C 27.	*Aerobacter* species	Isolated at Ardeer	A	34	6*	4·91	0·08
C 70.	*B. asiaticus-mobilis* Castellani	N.C.T.C. No. 1747	A	34	2	5·14	0·0
C 73.	*B. alkalescens* Andrewes	N.C.T.C. No. 1601	B	34	17	5·02	1·95
C 60.	*B. fecalis-alcaligenes* Petruschky (*Alcaligenes fecalis*)	N.C.T.C. No. 415	B	34	8	5·02	4·64
C 72.	*B. ureae* Leube	N.C.T.C. No. 1654	B	28	26	5·06	2·13
C 98.	*B. albolactis* (Löffler) Migula (298)	American Type Culture Collection, No. 3	B	28	17	5·22	2·29
C 67.	*B. mesentericus* No. 4	N.C.T.C. No. 2589	B	34	17	5·02	3·35
C 1.	*B. mesentericus*	Isolated at Ardeer	B	34	8	5·02	4·31
C 90.	*B. megatherium* De Bary	N.C.T.C. No. 654	B	34	10	4·77	3·92
C 30.	Rod bacillus, oval spores	Isolated at Ardeer	B	34	21	5·17	3·39
C 28.	*B. mycoides*	Isolated at Ardeer	B	34	21	5·17	2·86
C 69.	*B. bifermentans* Tissier and Martelly (*Clostridium bifermentans*)	N.C.T.C. No. 1340	B	34	15	4·77	3·38
C 96.	*Clostr. saccharobutyricum* Beijerinck	American Type Culture Collection, No. 860	B	34	7	5·17	1·47
C 94.	*Clostr. pasteurianum* Winogradsky	American Type Culture Collection, No. 861	B	34	7	5·17	1·39
C 80.	*Myc. phlei* Söhn.	N.C.T.C. No. 1471	B	34	21	5·17	4·23
C 82.	Moeller's "Mist" Bacillus (*Myc. stercusis*)	N.C.T.C. No. 526	B	34	17	5·02	3·97
C 81.	Bacillus (acid fast) from butter (butter bacillus of Rabinowitsch)	N.C.T.C. No. 524	B	34	8	5·02	4·64

N.C.T.C. refers to the British National Collection of Type Cultures, Lister Institute.

samples being withdrawn aseptically from time to time for sugar determination. Only if the sugar disappeared at a reasonable rate was it considered worth while to perform a complete metabolism experiment. In the event of the metabolism experiment being carried out, the control gave a useful indication as to the rate of progress of the fermentation, so that the correct time could be allotted for the incubation period.

Preliminary controls are lacking for some of the organisms used for the earlier balance sheets. In these cases the balance sheet figures are inserted in the table for the sake of completeness. Where this is done it is indicated by an asterisk. The results of the carbon balance sheet determinations are summarised in the Tables V–IX.

Table V.

	Leuconostoc species	*B. aroideae*	*B. caroto-vorus*	*B. proteus-vulgaris*	*B. proteus-vulgaris*
Medium used	B	B	B	B	B
Catalogue number	C 87	C 45	C 51	C 4	C 4
Temperature	34°	28°	28°	34°	34°
Incubation period in days ...	13	26	26	6	27
Carbon balance sheet (g.)					
Carbon in solution (start)	5·109	5·189	5·189	5·214	5·189
,, added as $CaCO_3$	0·840	0·837	0·839	0·838	0·835
Total carbon (start)	5·949	6·026	6·028	6·052	6·024
Carbon in H_2SO_4	0·002	0·007	0·003	0·002	0·007
,, in CO_2	0·651	1·324	0·576	0·383	0·435
,, in bacteria	0·100	0·233	0·059	0·081	0·167
,, in residual $CaCO_3$	0·450	0·579	0·450	0·625	0·527
,, in solution	4·679	3·786	4·848	4·941	4·840
,, accounted for	5·882	5·929	5·936	6·032	5·976
,, accounted for (%)	98·9	98·4	98·5	99·7	99·2
Analysis of solution (g.)					
Carbon in residual glucose	2·266	0·042	2·416	3·662	3·328
,, in CO_2 in solution	0·006	0·006	0·005	0·006	0·005
,, in volatile acids	0·457	0·288	0·475	0·344	0·313
,, in non-volatile acids (Ca ppt.)	0·146	0·336	0·323	0·084	0·117
,, in volatile neutral compounds	0·194	0·269	0·225	0·237	0·420
,, in synthetic compounds ...	0·063	0·119	0·101	0·057	0·053
,, as butylene glycol	0·311	2·136	Nil	—	0·168
,, as lactic acid	1·008	0·288	1·152	—	0·360
Total carbon accounted for	4·451	3·484	4·697	4·390	4·764
Total carbon in solution	4·679	3·786	4·848	4·941	4·840
Carbon unaccounted for (by difference)	0·228	0·302	0·151	0·550	0·076
Residual glucose (%)					
Glucose (by polarimeter)	0·828	Nil	1·187	1·774	1·653
,, (by Shaffer-Hartmann) ...	1·133	0·021	1·208	1·831	1·664
,, (by Wood-Ost)	1·104	0·021	1·182	1·805	1·692
Acids					
Titration (calculated) (cc. $N/1$) ...	65·0	43·0	64·8	35·5	51·3
Volatile acids (cc. $N/1$) ...	12·19	6·75	15·98	14·4	13·4
Barium salts (weight in g.)	2·050	1·083	2·584	1·996	1·692
Calcium salts (weight in g.)	0·451	1·020	0·918	0·104	0·098
Volume of oxygen absorbed (cc.) ...	395	590	350	93	210
Respiration coefficient	1·23	3·37	10·0	3·41	1·13
Volume of hydrogen evolved (cc.) ...	Nil	Nil	Nil	Trace	Nil

The bacterial strains tested in the control experiments (Table IV), and those employed in the complete metabolism experiments (Tables V to IX)

Table VI.

	B. acidi-lactici	*B. acidi-lactici* Groten-felt Hueppe	*B. acidi-lactici* (St Georges)	*B. coli* (*E. coli*)	*Escherichia* species from milk	*Escherichia* species from milk
Medium used	B	A	B	A	A	A
Catalogue number	C 54	C 6	C 62	C 34	C 37	C 26
Temperature	34°	34°	34°	34°	34°	34°
Incubation period in days ...	6	6	6	6	6	6
Carbon balance sheet (g.)						
Carbon in solution (start)	5·214	4·912	5·214	4·912	4·912	4·912
,, added as $CaCO_3$...	0·840	0·833	0·837	0·839	0·835	0·834
Total carbon (start)	6·054	5·745	6·051	5·751	5·747	5·746
Carbon in H_2SO_4	0·005	0·003	0·003	0·004	0·004	0·002
,, in CO_2	0·920	0·566	0·791	0·590	0·838	0·411
,, in bacteria	0·202	0·091	0·205	0·262	0·156	0·054
,, in residual $CaCO_3$	0·200	0·388	0·331	0·288	0·207	0·549
,, in solution	4·568	4·593	4·636	4·617	4·443	4·653
,, accounted for	5·895	5·641	5·966	5·761	5·648	5·669
,, accounted for (%)	97·4	98·2	98·6	100·2	98·3	98·6
Analysis of solution (g.)						
Carbon in residual glucose	1·101	2·190	2·030	2·508	1·092	3·121
,, in CO_2 in solution	0·008	0·006	0·003	0·004	0·005	0·004
,, in volatile acids...	0·524	0·253	0·719	0·434	0·659	0·381
,, in non-volatile acids (Ca ppt.)	0·311	0·263	0·097	0·105	0·115	0·045
,, in volatile neutral compounds	0·539	0·269	0·436	0·287	0·376	0·186
,, in synthetic compounds ...	0·074	0·052	0·032	0·040	0·047	0·038
,, as butylene glycol	Nil	Nil	Nil	0·022	0·042	0·072
,, as lactic acid	1·395	1·260	0·990	0·882	1·518	0·900
Total carbon accounted for	3·952	4·293	4·307	4·282	3·854	4·747
Total carbon in solution	4·568	4·593	4·636	4·617	4·443	4·653
Carbon unaccounted for (by difference)	0·616	0·300	0·329	0·335	0·589	—
Residual glucose (%)						
Glucose (by polarimeter)	0·600	1·280	0·988	1·345	0·662	1·539
,, (by Shaffer-Hartmann) ...	0·551	1·095	1·015	1·254	0·546	1·560
,, (by Wood-Ost)	0·585	1·128	1·019	1·290	0·598	1·525
Acids						
Titration (calculated) (cc. $N/1$) ...	106·7	74·2	84·4	91·9	104·7	47·5
Volatile acids (cc. $N/1$)	20·2	7·50	26·1	16·6	23·5	14·9
Barium salts (weight in g.)	2·882	1·331	4·019	2·400	3·457	2·182
Calcium salts (weight in g.)	0·812	0·772	0·055	0·034	0·146	—
Volume of oxygen absorbed (cc.) ...	119	13	131	125	66	77
Respiration coefficient	4·40	17·3	4·06	0·57	6·05	3·07
Volume of hydrogen evolved (cc.) ...	285	82·5	388	Nil	165	Nil

are listed by the names under which they were received, the source from which they were obtained being indicated in each case. No attempt has been made to study these strains from the systematic point of view; nor, in the present confused state of bacterial nomenclature, has it seemed desirable to insert alternative names, in accordance with any one of the systems in current use.

The strains brought together in Tables VI and VII are certainly closely related. Those in Tables V and IX include widely separated species. Our

Table VII.

	B. lactis-aero-genes	*B. lactis-aero-genes*	*A. cloacae*	*A. cloacae*	Leather bacillus Houston	*Aero-bacter* species from milk
Medium used	A	C	A	C	A	A
Catalogue number	C 24	C 24	C 12	C 12	C 25	C 27
Temperature	34°	34°	34°	34°	34°	34°
Incubation period in days	9	7	6	6	6	6
Carbon balance sheet (g.)						
Carbon in solution (start)	4·912	4·928	4·912	4·928	4·912	4·912
„ added as $CaCO_3$	0·835	—	0·831	—	0·836	0·839
Total carbon (start)	5·747	4·928	5·743	4·928	5·748	5·751
Carbon in H_2SO_4	0·019	0·004	0·010	0·005	0·004	0·006
„ in CO_2	1·677	0·864	1·131	0·478	1·251	1·254
„ in bacteria	0·256	0·090	0·183	0·003	0·168	0·138
„ in residual $CaCO_3$	0·693	—	0·504	—	0·306	0·371
„ in solution	2·750	3·784	3·842	4·494	3·948	3·885
„ accounted for	5·395	4·742	5·670	4·980	5·677	5·654
„ accounted for (%)	93·9	96·2	98·7	101·0	98·8	98·3
Analysis of solution (g.)						
Carbon in residual glucose	0·017	1·366	0·863	2·768	0·081	0·083
„ in CO_2 in solution	0·008	0·006	0·006	0·006	0·006	0·006
„ in volatile acids	0·155	0·106	0·326	0·198	0·648	0·618
„ in non-volatile acids (Ca ppt.)	0·062	0·341	0·104	0·483	0·072	0·030
„ in volatile neutral compounds	0·687	0·518	0·684	0·362	0·604	0·527
„ in synthetic compounds	0·020	0·008	0·016	0·003	0·057	0·048
„ as butylene glycol	1·527	0·912	1·603	0·465	0·661	0·968
„ as lactic acid	0·157	0·504	0·128	0·072	1·710	1·710
Total carbon accounted for	2·633	3·761	3·730	4·357	3·839	3·990
Total carbon in solution	2·750	3·784	3·842	4·494	3·948	3·885
Carbon unaccounted for (by difference)	0·117	0·023	0·112	0·137	0·109	—
Residual glucose (%)						
Glucose (by polarimeter)	0·113	0·773	0·462	1·418	0·275	0·243
„ (by Shaffer-Hartmann)	0·008	0·683	0·432	1·384	0·203	0·042
„ (by Wood-Ost)	—	0·686	0·435	1·378	0·192	0·038
Acids						
Titration (calculated) (cc. $N/1$)	23·6	15·0	54·6	8·0	88·3	77·9
Volatile acids (cc. $N/1$)	—	3·37	20·3	6·88	18·4	12·8
Barium salts (weight in g.)	0·085	0·450	2·579	1·195	2·902	2·367
Calcium salts (weight in g.)	0·114	4·108	0·155	4·539	—	—
Volume of oxygen absorbed (cc.)	652	104	117	58	217	151
Respiration coefficient	4·4	15·4	12·8	15·4	6·2	9·7
Volume of hydrogen evolved (cc.)	392	Trace	Nil	Nil	262	196

purpose, in these preliminary experiments, has been to demonstrate the applicability of this method to the study of bacterial metabolism, and to cover a wide range of species or genera before selecting some of them for more detailed systematic study.

Table VIII.

	B. asiaticus-mobilis (Cast)	*B. asiaticus-mobilis* (Cast)	*B. alkalescens* (Andrewes)
Medium used	A	A	B
Catalogue number	C 70	C 70	C 73
Temperature	34°	34°	34°
Incubation period in days ...	2	2	41
Carbon balance sheet (g.)			
Carbon in solution (start)	4·912	4·912	5·191
„ added as $CaCO_3$...	0·840	0·838	0·840
Total carbon (start)	5·752	5·750	6·031
Carbon in H_2SO_4	0·010	0·012	0·003
„ in CO_2	1·140	1·451	0·540
„ in bacteria	0·233	0·248	0·190
„ in residual $CaCO_3$	0·613	0·585	0·236
„ in solution	3·217	3·407	4·870
„ accounted for	5·213	5·703	5·839
„ accounted for (%)	90·6	99·2	96·8
Analysis of solution (g.)			
Carbon in residual glucose	0·013	0·000	1·960
„ in CO_2 in solution	0·006	0·006	—
„ in volatile acids...	0·588	0·326	0·515
„ in non-volatile acids (Ca ppt.)	0·142	0·097	0·221
„ in volatile neutral compounds	0·941	0·984	0·122
„ in synthetic compounds ...	0·065	0·185	0·102
„ as butylene glycol	1·339	1·551	0·380
„ as lactic acid	Nil	Nil	1·300
Total carbon accounted for	3·094	3·149	4·600
Total carbon in solution	3·217	3·407	4·870
Carbon unaccounted for (by difference)	0·123	0·258	0·270
Residual glucose (%)			
Glucose (by polarimeter)	0·113	0·162	1·191
„ (by Shaffer-Hartmann) ...	0·007	Nil	0·980
„ (by Wood-Ost)	—	Nil	0·860
Acids			
Titration (calculated) (cc. *N*/1) ...	37·9	42·2	100·8
Volatile acids (cc. *N*/1)	8·54	6·49	17·4
Barium salts (weight in g.)	2·064	1·203	2·614
Calcium salts (weight in g.)	0·436	0·215	0·626
Volume of oxygen absorbed (cc.) ...	79	89	580
Respiration coefficient	21·6	25·1	—
Volume of hydrogen evolved (cc.) ...	308	430	Nil

Review of balance sheets.

The figures given in parentheses below are taken direct from the balance sheets and denote the weight of carbon, in the class of products mentioned, which is obtained from 5 g. of carbon as glucose. Hence the approximate yield % is obtained by multiplying the quoted figure by 20.

Leuconostoc. The species employed gives a high yield of lactic acid (1·008) and moderate yields of volatile acids (0·457) and of butylene glycol (0·311), considering that nearly half the original sugar remains unfermented.

B. aroideae and *B. carotovorus.* The balance sheets for these two species

Table IX.

	B. ureae Leube (*Urobacillus*)	*B. albo-lactis*	*Clostridium saccharo-butyricum*	*Clostridium pasteurianum*
Medium used	B	B	B (anaerobic)	B (anaerobic)
Catalogue number	C 72	C 98	C 96	C 94
Temperature	28°	28°	34°	34°
Incubation period in days ...	27	27	7	9
Carbon balance sheet (g.)				
Carbon in solution (start)	5·189	5·189	5·477	5·315
„ added as $CaCO_3$...	0·839	0·839	1·681	1·666
Total carbon (start)	6·028	6·028	7·158	6·981
Carbon in H_2SO_4	0·001	0·001	0·001	0·003
„ in CO_2	0·650	0·633	1·211	1·198
„ in bacteria	0·157	0·155	0·506	0·509
„ in residual $CaCO_3$	0·555	0·336	1·123	1·080
„ in solution	4·641	4·781	4·000	3·903
„ accounted for	6·004	5·906	6·841	6·693
„ accounted for (%)	99·6	98·0	95·6	95·9
Analysis of solution (g.)				
Carbon in residual glucose	2·486	1·840	1·346	1·538
„ in CO_2 in solution	0·005	0·006	0·006	0·006
„ in volatile acids...	0·287	0·532	1·892	1·744
„ in non-volatile acids (Ca ppt.)	0·184	0·081	0·104	0·121
„ in volatile neutral compounds	0·136	0·066	0·145	0·291
„ in synthetic compounds ...	0·061	0·075	0·109	0·126
„ as butylene glycol	0·841	0·062	—	—
„ as lactic acid	0·756	1·530	—	—
Total carbon accounted for	4·756	4·202	3·602	3·826
Total carbon in solution	4·641	4·781	4·000	3·903
Carbon unaccounted for (by difference)	0·115	0·579	0·398	0·077
Residual glucose (%)				
Glucose (by polarimeter)	0·911	0·563	0·583	0·664
„ (by Shaffer-Hartmann) ...	1·243	0·920	0·673	0·769
„ (by Wood-Ost)	1·206	0·900	0·664	0·710
Acids				
Titration (calculated) (cc. *N*/1) ...	47·3	83·8	93·0	97·7
Volatile acids (cc. *N*/1)	7·80	16·0	49·3	45·7
Barium salts (weight in g.)	1·070	2·590	12·51	10·65
Calcium salts (weight in g.)	0·537	Nil	0·162	0·216
Volume of oxygen absorbed (cc.) ...	430	470	Nil (anaerobic)	Nil (anaerobic)
Respiration coefficient	1·59	0·52	—	—
Volume of hydrogen evolved (cc.) ...	Nil	Nil	1263	1299

show considerable differences. *B. aroideae*, C 45, gives a very high yield of butylene glycol (2·136) equivalent to over 40 % of the original sugar, and very little lactic acid (0·288), whereas *B. carotovorus*, C 51, gives no butylene glycol and a high yield of lactic acid (1·152), corresponding to about 40 % on the sugar consumed.

B. proteus-vulgaris. In the first balance sheet the fermentation had not proceeded far although the incubation period was 6 days; therefore butylene

glycol and lactic acid were not determined. A second balance sheet experiment was performed in which the culture was incubated for 27 days. It was found that very little further fermentation had taken place, although the culture was still viable. With this organism, up to 30 % of the original sugar disappears in the early stages, while very little subsequent change is obvious. The early cessation of fermentation may be due to the exhaustion of one of the nutrients of the medium or to inhibition by products of bacterial metabolism.

B. acidi-lactici and *B. coli group*. This group is notable for the fact that it produces large amounts of lactic acid (up to 1·52) but negligible amounts of butylene glycol. A fair amount of volatile acid is produced (0·25–0·72), and a smaller amount of volatile neutral compounds (0·19–0·54). Too much importance should not be attached to the volatile acid determination, owing to the presence of large amounts of lactic acid. For the same reason, the "carbon unaccounted for" will tend to be low. The most interesting point is the fact that the "carbon unaccounted for" is not negligible; in two cases, it reaches the figures of 0·62 and 0·59 respectively, although 20 % of the original sugar still remains, and in spite of the fact that the "carbon unaccounted for" figure is probably too low, for the reason given above. These two species C 54 and C 37 are marked out for further investigation.

Aerobacter. This group produces lactic acid and butylene glycol. All grades are shown from a high yield of lactic acid (1·71) and a moderately low yield of butylene glycol to a low yield of lactic acid and a high yield of butylene glycol (1·60). In all cases, a fair amount of volatile neutral substances (probably ethyl alcohol) is formed (0·36–0·68). In no case is there any appreciable amount of "carbon unaccounted for". The balance sheets for *B. lactis-aerogenes* and *A. cloacae* bear out the results obtained by Harden *et al.* [1906; 1912, 1, 2].

The *B. asiaticus-mobilis* Castellani shows a remarkably rapid fermentation, the sugar in a 5 % solution being completely metabolised in less than 48 hours. The chief characteristic is the high yield of butylene glycol (1·34 and 1·55) which would make the organism specially suitable for the preparation of this glycol. A considerable amount of alcohol (0·94, 0·98) is formed, and a fair amount of volatile acid (0·59, 0·33). No lactic acid is produced, and the carbon unaccounted for is negligible.

A quantity of butylene glycol was prepared in the laboratory from the fermentation of glucose by *B. asiaticus-mobilis*. The fermentation was carried out in 750 cc. and 1 litre flasks containing 250 cc. and 350 cc. of medium respectively, also in 5 litre flasks containing 4 litres of medium. There was little difference in the yield obtained in the two cases, but the fermentation period was longer in the large flasks, due probably to the settling of the calcium carbonate with less rapid neutralisation of the acid produced. This effect was naturally less pronounced when the liquid was distributed in a shallow layer. Aeration had little or no effect on the fermentation, but as it provided a means of agitation of the carbonate it was advantageous from this point of view.

The best yields were obtained from a comparatively slow fermentation (7 days) occurring in presence of heavy carbonate and representing a 28–29 % recovery. No explanation is offered for this somewhat curious result.

The fermentation of 5 % sugar solution in small flasks occupied 2 days, but with higher concentrations of sugar longer periods were required—thus $12\frac{1}{2}$ % sugar with increased amount of carbonate present needed 14 days for complete fermentation.

The method of recovery of the butylene glycol was very simple. The solution was filtered from undissolved calcium carbonate, evaporated to small bulk *in vacuo* and finally distilled *in vacuo*. Usually the solid residue remaining after distillation was washed with alcohol and a further small amount of the glycol recovered in this way. The total amount of butylene glycol recovered was 2435 g. representing an average yield of 22·3 % on the sugar fermented. The results of the experiments are summarised in Table X.

Table X. *Production of butylene glycol.*

Time in days	Initial sugar %	Initial sugar Weight in g.	Final sugar %	Final sugar Weight in g.	Sugar fermented g.	Glycol recovered g.	% yield	
5	5	25	0·21	1·0	24			
25	10	50	Nil	Nil	50	20	20·2	—
20	5	25	Nil	Nil	25			
4	5	1750	Nil	Nil	1750			
7	5	200	0·22	8·8	191	314	15·5	—
4	5	87·5	Nil	Nil	88			
6	5	1663·5	0·28	93·2	1570	450	28·6	Heavy $CaCO_3$
2	5	1750	0·45	157·5	1592	287	18·0	—
8	5	1200	0·40	96·0	1104	261	23·7	—
3	5	1750	Nil	Nil	1750			
7	5	200	0·32	12·8	187	496	25·6	—
7	5	440	Nil	Nil	440	126	28·6	Heavy $CaCO_3$
2	5	600	Nil	Nil	600	125	20·8	Aerated
4	5	400	0·22	17·6	382	87	22·8	Non-aerated
2	7·5	300	0·42	16·8	283	68	24·0	Aerated
6	10	400	Nil	Nil	400	89	22·3	Aerated
14	12·5	500	0·2	8	492	112	22·8	Aerated
				Total	10,928	2435	22·3	

B. alkalescens Andrewes, C 73. This organism produces a large amount of lactic acid (1·30), considerably smaller amounts of butylene glycol (0·38) and volatile acids (0·515). The carbon unaccounted for is small in amount.

B. ureae and *B. albolactis*. These two organisms show several points of interest. *B. ureae* (C 72) shows a small surplus in the balance sheet which is most unusual though possibly accounted for by the volatility of lactic acid. *B. albolactis* (C 98) on the other hand shows a "carbon unaccounted for" figure of 0·58, which is considerable in view of the amount of unfermented glucose remaining. The amount of "non-volatile acids" is low in both cases, but a fair amount of "volatile acids" is formed, particularly in the case of C 98. There is a curious discrepancy in both cases between the glucose determinations, the polarimeter figure being considerably lower than that obtained

by copper reduction. This appears to indicate the formation of some laevo-rotatory compound. *B. albolactis* thus merits further investigation.

Clostridium. These anaerobic species are remarkably similar in behaviour. The characteristic is the production of a large amount of volatile acids (1·89 and 1·74). Butylene glycol and lactic acid were not determined, but could only be present in small amount, since the "carbon unaccounted for" is low.

Discussion.

In performing these balance sheet experiments the time allotted for each fermentation varies since the time was chosen according to the rate of fermentation. An endeavour was made in each case to interrupt the experiment at such a point that most, but not all, of the sugar had disappeared, so that the amount of intermediate metabolic products should be as large as possible. This involves wide differences in the incubation periods of the various organisms. In cases where the fermentation is slow, there is a greater likelihood of bacterial autolysis. This introduces the possibility of carbohydrate breakdown or attack on the intermediate products by the enzymes liberated by cell autolysis, in addition to the coordinated fermentative action of these enzymes when under the control of the living cell. This difficulty cannot be entirely obviated in work of this type, but it would be advisable in future experiments to determine growth curves for each organism studied, in order to correlate the fermentation during the rapid growth period with that occurring during the autolysis period, as indicated by a decline in the viable count.

The possibility of autolysis raises an interesting point in the interpretation of the balance sheets. The "carbon in bacteria" will not represent the total amount of carbon utilised to form cell-matter if a portion of the cells autolyses in the later stages of fermentation. However, it appears likely that a large proportion of the autolysis products will, in such cases, be found in the "carbon in synthetic products", *i.e.* in the carbon compounds precipitated by "colloidal iron."

It has been shown that the quantitative method for the classification of the types of metabolic products of micro-organisms although hitherto used only for moulds, applies equally well to bacteria provided certain minor modifications are made. The chief difficulty consists in finding a suitable synthetic medium containing no carbon apart from the glucose supplied for fermentation. Although some of the organisms were able to grow and ferment on such a medium, the number was found to be very limited, and a much wider field became available by the introduction of small amounts of organic nitrogen in the form of peptone. This is justifiable so long as the amount of carbon thus introduced is small in relation to the carbon present as glucose.

Reference has already been made to the fact that addition of calcium carbonate to the medium was found more generally useful as a method of neutralising the acidity formed than the use of large amounts of phosphate.

In the case of two organisms, *Aerobacter aerogenes* and *cloacae* (C 24 and C 12), comparative balance sheets were obtained using the carbonate and the phosphate buffer media. A study of these reveals the fact that although fermentation was retarded on the phosphate medium, the products were not widely different in nature, the chief difference being a considerable increase of the non-volatile acid fraction with excess of phosphate. This may be due to the formation of hexosephosphate. In a third case—that of *B. acidi-lactici* (Hueppe), C 6, on the phosphate medium practically no gas was liberated during the 6 days of the experiment, only about half the sugar had disappeared and at the end of the period the organism was apparently dead. The balance sheet is not given. On the carbonate medium, the fermentation was definitely faster and was still in progress at the end of 6 days. The phosphate buffering appears inadequate when the organism produces a large amount of acid, as in the case of C 6.

Many of the bacteria appear to utilise glucose merely for growth purposes. This is shown by the large number of control experiments in which only a small proportion of the sugar disappeared. These organisms cannot therefore be said to ferment the sugar in the usual sense of the term.

We may now compare briefly the types of products obtained from bacteria with those derived from moulds. The mould products (apart from mycelium and carbon dioxide) group themselves chiefly in the classes "volatile neutral substances" (ethyl alcohol), "non-volatile acids" and "carbon unaccounted for", the last named, when large in amount, often consisting of mannitol or glycerol. Only traces of volatile acids are produced. Lactic acid and butylene glycol have never been reported as mould metabolic products. With moulds, on the other hand, various synthetic substances such as polysaccharides, sterols, pyrones, quinonoid and phenolic substances, complex fatty acids, *etc.*, are formed.

The main products of bacterial metabolism are "volatile neutral substances", "volatile acids" and much smaller amounts of "non-volatile acids". Lactic acid and butylene glycol are of frequent occurrence and are often produced in considerable quantity. Thus species of *Aerobacter* give varying amounts of both products, of *Escherichia* give considerable amounts of lactic acid but little butylene glycol, and of *B. asiaticus mobilis* (Cast.) large amounts of butylene glycol but no lactic acid.

Hydrogen is a frequent product of bacterial action, particularly in the case of anaerobes, but has never been obtained from moulds.

Although polysaccharides are known to be formed [Grey, 1924], no evidence has yet been obtained of any phenolic substances such as are elaborated by moulds.

The products of bacterial metabolism have been shown to be, in general, more volatile than those of moulds. Thus, volatile acids replace the non-volatile acids, butylene glycol is obtained instead of mannitol or glycerol, and so on. This may be due to the fact that moulds are obligate aerobes, whilst many bacteria are facultative anaerobes, and consequently mould

metabolic products are more highly oxidised. It is well known that an increase in the oxygen content of organic substances (as hydroxyl or carboxyl groups) lowers their volatility. In this connection it is interesting to note [Hermann and Neuschul, 1931] that eight strains of acetic acid bacteria, the reactions of which are almost purely oxidative and only feebly hydrolytic, give products such as gluconic acid, substances which are more akin to those of mould metabolism.

The oxidative nature and greater synthetic activity of mould fermentation may be said to be the chief characteristics which differentiate it from bacterial fermentation.

Summary.

The Czapek-Dox medium as used for the determination of mould metabolism products by the balance sheet method has been modified so as to render it suitable for the cultivation of bacteria. This involves the substitution of the sodium nitrate of the original medium by microcosmic salt of equivalent nitrogen content. The number of organisms showing growth and fermentation on this medium was small but was greatly increased by addition also of 0·2 % peptone, an amount insufficient to interfere appreciably with the carbon balance sheet estimations. Calcium carbonate proved to be the most useful buffering agent. Certain modifications necessary when applying the standard balance sheet technique to bacteria are detailed, and methods employed for the determination of 2 : 3-butylene glycol and lactic acid, which are of frequent occurrence, are described.

Fifty species of bacteria were found to grow readily on the selected medium and of these, 20 species were chosen for examination by the balance sheet method at 28° and 34°. The balance sheets show that the chief classified products are volatile acids, volatile neutral substances, lactic acid and 2 : 3-butylene glycol.

Of these organisms, *B. asiaticus-mobilis* proved interesting in that it gave about a 30 % yield of butylene glycol and the rate of fermentation was faster than for any of the other organisms examined, whilst the balance sheets of three other organisms (*B. acidi-lactici*, C 54, *Esch.* species from milk, C 37, and *B. albolactis*, C 98) indicate that these species merit further investigation.

We greatly appreciate the interest taken in this work by Professors H. Raistrick and W. W. C. Topley, and our best thanks are due to them for their advice and helpful criticism. We are also indebted to Mr W. Rintoul and the Directorate of Imperial Chemical Industries for permission to publish this work.

REFERENCES.

Birkinshaw and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 11.
von Fürth and Charnass (1910). *Biochem. Z.* **26**, 199.
Grey (1924). *Biochem. J.* **18**, 712.
Harden and Norris (1912, 1). *Proc. Roy. Soc. Lond.* B **84**, 492.
—— —— (1912, 2). *Proc. Roy. Soc. Lond.* B **85**, 73.
Harden and Walpole (1906). *Proc. Roy. Soc. Lond.* B **77**, 399.
Hermann and Neuschul (1931). *Biochem. Z.* **233**, 129.
Malaprade (1928). *Compt. Rend. Acad. Sci.* **186**, 382.
Raistrick and co-workers (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 27, 55, 93, 99.

CLXVII. ON THE GLYOXALASE-CO-ENZYME RATIO IN LIVER TISSUE IN INANITION[1].

By PAUL VOGT-MØLLER.

From the County and City Hospital, Medical Department, Odense, Denmark.

(*Received August 7th, 1931.*)

In a previous communication [Vogt-Møller, 1931] on the glyoxalase-co-enzyme ratio in liver tissue in avitaminosis $B_1 + B_4$ [Reader, 1930], *i.e.* in experimental beriberi in mice, it was stated that starved animals, from which food was withdrawn, but which were allowed to take water, differed from animals suffering from avitaminosis $B_1 + B_4$, whose allowance of food was complete in every respect with the exception of vitamins B_1 and B_4, in such a manner that the liver tissue of the former animals (and probably the whole organism except the pancreas, in which special conditions prevail) in contradistinction to that of the latter contained more co-enzyme (co-mutase), though in both cases less than the normal amount was present. Under the conditions of the experiments, therefore, methylglyoxal was partly stabilised in avitaminous ferment systems.

When the technique that was then used is employed, *viz.* the determination of acidity described by Neuberg [1913, 1, 2] and by Dakin and Dudley [1913, 1, 2, β], the precipitation of methylglyoxal employed by Vogt [1929], either as *p*-nitrophenylosazone or as *bis*-2 : 4 dinitrophenylhydrazone (described by Neuberg and Kobel [1928]), and the colorimetric method described by Ariyama [1928], all the methods being employed after 24 (48) hours' incubation of the ferment system at 37°, and when the technique used by me for the institution of inanition is used, *i.e.* the food but not the water is removed from the cage, the floor of which is covered with wood-wool and sawdust, the facts previously stated still hold good, provided the inanition is not carried too far.

Having begun to use Ariyama's method, but only with 20 minutes' incubation, and letting the animals starve in glass cages devoid of shavings instead of in the manner referred to above, I have attained results which deviate somewhat from those previously communicated, for which reason they are now briefly published.

As to the quantitative (colorimetric) determination of methylglyoxal I refer to Ariyama's communication. The incubation time was only 20 minutes at 37°, and by determining the amount of the added methylglyoxal which has been used during this period a measure of the glyoxalase + co-enzyme

[1] Supplementary note to my article in *Biochem. J.* (1931), **25**, 418.

content of the organ (in this case of the liver) is obtained. As glyoxalase + co-enzyme preparation I used a 20 % preparation of ground liver tissue suspended in distilled water after it had been allowed to stand for 1 hour at room temperature after evisceration and after filtration of the suspended tissue. As co-enzyme preparation I used a 10 or 20 % boiled (5 minutes) preparation of suspended liver tissue prepared as stated above. Co-enzyme-free preparations were prepared by means of 6 hours' dialysis of the glyoxalase + co-enzyme preparations.

Using this method it appeared that an inanition of short duration (42 hours in the case of mice) involved a very considerable loss of the co-enzyme content of the liver tissue. It is a well-known though remarkable fact that the co-enzyme is used up during the fermentation process *in vitro*; the same thing may also occur *in vivo*. In contradistinction to this fact Ariyama found that after 10 days' inanition the amount of glyoxalase (*i.e.* glyoxalase + co-enzyme) in liver tissue (rabbit) "remained unchanged," whilst Sakuma [1930] found that 10 days' inanition of a rabbit caused a considerable decrease of the amount of co-enzyme.

In relation to what has been communicated previously, *viz.* the abnormal conditions of carbohydrate metabolism in avitaminosis B_1, I tried, in relation to the glucose cure [Kinnersley *et al.* 1928], the possible co-enzyme-forming or co-enzyme-stimulating power of glucose (saccharum amylaceum puriss. anhydric. U.S.P.X.) and found, like Sakuma, that weak solutions of glucose *in vitro* furthered the dismutation of methylglyoxal in dialysed, *i.e.* co-enzyme-free, ferment systems, and similar conditions were also found in the case of weak solutions of sodium nucleinate (originating partly from yeast, partly from the thymus gland). This fact in view of Euler and Myrbäck's [1929] supposition that co-enzyme (co-mutase) is "ein Adenin-nucleotid besonderer Art" brings the co-enzyme in a rather close relation to vitamin B_1 or perhaps especially vitamin B_4, without, however, allowing us to identify these two substances. One might suppose that the vitamin contained constituents necessary for the composition or formation of the co-enzyme. This much is certain: in inanition and in avitaminosis $B_1 + B_4$ there is a decrease of the amount of co-enzyme, a decrease that is not found in the controls used (paired-feeding method). It is possible that the cause of the decrease of the co-enzyme concentration in avitaminosis $B_1 + B_4$ should be looked for in the anorexia, always engendered by the avitaminosis, although this is not very probable when the conditions of the controls are considered, but it is now at least within our reach to find out the substances (nutritives, chemical compounds) causing the formation of co-enzyme *in vivo*. Possibly the toxic effect of glucose injected parenterally in excess is best understood on the ground that by the catabolism of glucose the tissue is deprived of the co-enzyme.

SUMMARY.

Information is given about the decrease of the co-enzyme concentration of liver tissue in inanition and about the co-enzymic properties of glucose and sodium nucleinate. Attention is drawn to the fact that it is now possible to examine the co-enzyme formation *in vivo*.

REFERENCES.

Ariyama (1928). *J. Biol. Chem.* **77**, 359.
Dakin and Dudley (1913, 1). *J. Biol. Chem.* **14**, 155.
—— —— (1913, 2). *J. Biol. Chem.* **14**, 423.
—— —— (1913, 3). *J. Biol. Chem.* **15**, 463.
Euler and Myrbäck (1929). *Z. physiol. Chem.* **184**, 163.
Kinnersley, Peters and Reader (1928). *Biochem. J.* **22**, 276.
Neuberg (1913, 1). *Biochem. Z.* **49**, 502.
—— (1913, 2). *Biochem. Z.* **51**, 484.
—— and Kobel (1928). *Biochem. Z.* **203**, 463.
Reader (1930). *Biochem J.* **24**, 1827.
Sakuma (1930). *J. Biochem.* **12**, 247.
Vogt (1929). *Biochem. Z.* **211**, 17.
Vogt-Møller (1931). *Biochem. J.* **25**, 418.

CLXVIII. THE REDUCTION OF NITRATES BY *BACT. COLI.*

By LEONARD HUBERT STICKLAND[1].

From the Biochemical Laboratory, Cambridge.

(*Received July 31st, 1931.*)

Introduction.

It has long been known that many bacteria are able to reduce nitrates to nitrites, and this fact is extensively used in classification. More exact investigation of the process is of recent date. Quastel, Stephenson and Whetham [1925] showed that nitrate could produce anaerobic growth of some facultative anaerobes in media which without it would not support growth, *i.e.* the nitrate could take the place of molecular oxygen. The coupled reaction, with reduction of nitrate to nitrite, always yields less energy than the corresponding direct oxidation, *e.g.*,

$$\text{lactic acid} + KNO_3 \rightarrow \text{pyruvic acid} + H_2O + KNO_2 + 30{\cdot}7 \text{ cals.}$$

$$\text{lactic acid} + \tfrac{1}{2}O_2 \rightarrow \text{pyruvic acid} + H_2O + 51{\cdot}9 \text{ cals.}$$

Quastel and Whetham [1924] showed that the utilisation of nitrate as a hydrogen acceptor depended on the power of the organism to activate the nitrate molecule, by demonstrating that nitrates oxidise leucomethylene blue in the presence of a washed suspension of cells but not in its absence. The capacity of a species of bacteria for anaerobic growth on, say, lactate and nitrate, is, of course, parallel to its power of oxidising leucomethylene blue by nitrate.

In its rôle as a supporter of growth in media containing only non-fermentable carbon compounds nitrate is strictly comparable with molecular oxygen, and the object of the work described here is to make a comparison between the reduction of nitrate to nitrite and that of oxygen to water.

In considering the enzymic mechanisms involved, there appear to be three possibilities, *viz.*,

(1) the activations of oxygen and nitrate are carried out by the same enzyme;

(2) the enzymes concerned are different, and specific for oxygen and nitrate respectively;

(3) the activation of nitrate is carried out by an accessory mechanism in

[1] Goldsmiths' Senior Student and Benn W. Levy Student.

close connection with the oxygen-activating enzyme. This last is a very vague conception, which may have to be considered if the other hypotheses fail.

Methods.

The organism chosen was *Bact. coli* (Escherich); two different preparations of the cells were used, first an ordinary washed and aerated suspension, and second the same suspension treated with toluene at room temperature [Quastel and Wooldridge, 1927; Cook, 1930]. For different purposes the one or the other proved more convenient, but wherever the same experiment could be carried out with both, essentially similar results were obtained. The chief differences of behaviour produced by toluene treatment are (1) the endogenous respiration of the cells is reduced to negligible proportions, *i.e.* methylene blue, oxygen and nitrate are not reduced except in the presence of an added donator such as hydrogen, formate, lactate, *etc.*, (2) the oxidation of lactate and succinate proceeds only as far as pyruvate and fumarate respectively, and (3) the suspension is incapable of attacking glucose.

The course of the reduction of nitrate was followed by two methods. The first consisted of placing the cell suspension in the cups of a Barcroft differential manometer, with a measured amount of nitrate in the right-hand cup, filling the apparatus with hydrogen, and observing the rate at which hydrogen was absorbed [*cf.* Stephenson and Stickland, 1931]. The other method was carried out by keeping the suspension anaerobic in the presence of a known concentration of nitrate and of hydrogen donator (*e.g.* lactate) and estimating the nitrite produced, at various time intervals, colorimetrically by means of the Griess-Ilosvay reagent. The most convenient apparatus for this purpose was the ordinary Thunberg tube. A set of tubes containing nitrate, donator (usually lactate), buffer and cell suspension was evacuated and placed in a bath, and for each reading one tube was taken out and the concentration of nitrite in it estimated.

To prepare the reagent completely colourless the sulphanilic acid and α-naphthylamine were recrystallised from water and light petroleum respectively, dissolved in 50 % acetic acid made with glass-distilled water, and mixed in equal proportions when required. Glass-distilled water was used in making up all solutions, as the ordinary distilled water often contained appreciable amounts of nitrite. The appropriate quantity of the solution to be estimated was added to 5 cc. of the reagent, and made up to 20 cc. with 50 % acetic acid. The standard was a 0·0001 *M* solution of pure recrystallised silver nitrite, of which 1 or 2 cc. were used. The maximum colour developed in 15–20 minutes and was stable for at least 24 hours. By this method duplicate tubes always agreed to within 3 %. All the experiments were carried out at 40° in the presence of phosphate buffer at p_H 7·0, except where otherwise stated.

Preliminary observations.

(1) *The extent of reduction of nitrate.* With toluene-treated cells, using the Barcroft manometer method, the hydrogen taken up corresponded to 90–100 % of the theoretical for reduction to nitrite, *e.g.*

	Amount of KNO_3	H_2 uptake mm.^{3}	Theoretical	%
1	0·5 cc. *M*/50	227	224	101
2	,,	210	224	94
3	,,	215	224	96

Other donators, added in excess to known amounts of nitrate in Thunberg tubes, with either normal or toluene-treated suspensions, also gave 100 % reduction as measured by nitrite estimations, *e.g.* with lactate,

	KNO_3 added *M*	Nitrite found *M*	%
1	0·0250	0·0245	97·5
2	0·0200	0·0197	98·5
3	0·0200	0·0198	99

With untreated suspensions, the hydrogen uptake by the first method always fell short of the theoretical, owing to reduction of part of the nitrate by other donators in the cells [see Stephenson and Stickland, 1931], but estimation of nitrite showed the reduction to be complete.

(2) *The extent of oxidation of hydrogen donators.* Cook and Stephenson [1928] showed that with oxygen as the hydrogen acceptor an untreated suspension of *Bact. coli* oxidised formate completely, while with lactate the oxygen uptake was two-thirds of that required for complete oxidation, and with succinate there was a rapid oxidation as far as fumarate with an indefinite and slower further oxidation. It has been impossible to get parallel results with nitrate, owing to the great rapidity with which the untreated cells reduce nitrate in the absence of added donators.

In the case of toluene-treated suspensions, Quastel and Wooldridge [1927] and Cook [1930] have shown that formate is completely oxidised in oxygen,

Donator	Conc. of donator *M*	Conc. of NO_2^- *M*	Extent of oxidation %	Average %	O_2 uptake (Cook) %
Lactate	0·004	0·00332	83		
		0·00353	88		
		0·00317	79	82	78
	0·002	0·00159	80		
		0·00162	81		
Succinate	0·004	0·00312	78		
		0·00337	84		
		0·00303	76	82	82
	0·002	0·00168	84		
		0·00178	89		
Formate	0·0051	0·00472	93		
		0·00461	90		
		0·00472	93	92	98
		0·00465	91		
	0·00255	0·00238	93		
		0·00231	91		

while lactate and succinate go as far as pyruvate and fumarate respectively. The results for reduction of nitrate were obtained by incubating tubes containing a measured amount of the donator and excess of nitrate until there was no further increase in the nitrite concentration. The results are given above, with Cook's [1930] figures for the percentage of the theoretical oxygen uptake in the last column. The oxidations are incomplete, but proceed as far as they do with oxygen.

(3) *The linearity of the reaction.* Starting with 0·02 *M* potassium nitrate and 0·02 *M* lactate, and using a dilute suspension of cells, the initial course of the reaction may be determined while less than 1 % of the reaction is taking place, and the rate was found to be constant within the limits of accuracy of the method (Fig. 1). With a thicker suspension the whole course of the reaction may be followed, as in Fig. 2.

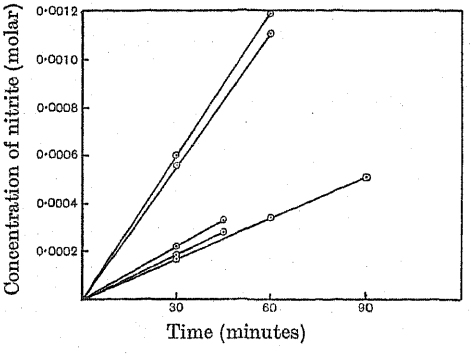


Fig. 1.

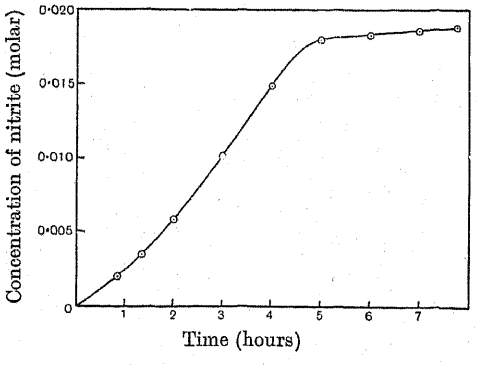
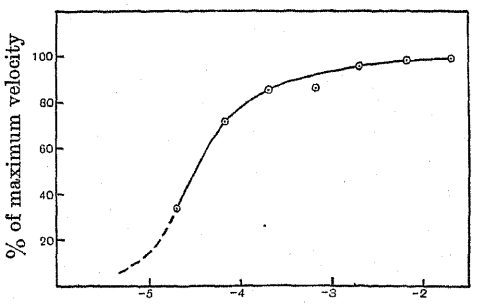


Fig. 2.

(4) *The affinity of the enzyme for nitrate.* The initial velocity of the reaction was measured at a series of nitrate concentrations by the vacuum tube method. No great decrease in velocity with diminishing concentration was observed until concentrations of nitrate were reached at which the method of estimation

of the nitrite had reached its limit, so the lowest points on the curve (Fig. 3) are not very accurate; it was possible, however, to make an estimate of the



Logarithm of molar concentration of nitrate

Fig. 3.

affinity, and the three separate values obtained for the Michaelis constant were 0·00005, 0·00003 and 0·00005 *M*. Hence one can be sure of being able to get initial velocity values at concentrations of nitrate down to at least 0·0005 *M*.

(5) *The effect of* p_H *on the velocity.* Using the vacuum tube method for measuring the rates, the curves shown in Fig. 4 were obtained, giving the

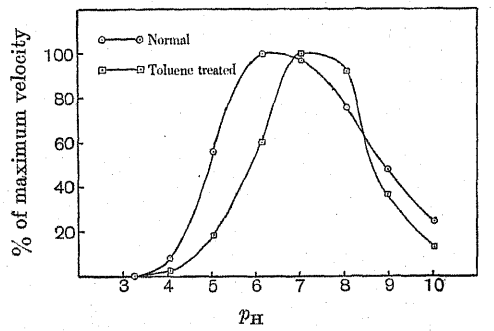


Fig. 4.

relation between p_H and initial velocity of nitrate reduction. Treatment with toluene causes a marked change in the p_H curve. Both curves are given as percentages of the maximum velocity, but the actual optimum with the toluene-treated suspension is only about 60–70 % of that before treatment, so that the apparent acceleration at p_H 8 is not real.

(6) It was also shown that variation of the lactate concentration within narrow limits about 0·02 *M* caused no great difference in the reaction velocity:

Lactate conc. (*M*)	Initial velocity (*M* NO_2^- per hour)
0·02	0·00081
0·0067	0·00079
0·002	0·00069

As a consequence of these preliminary results, the following was used as a standard method.

1 cc. 0·1 *M* potassium nitrate, 1 cc. 0·1 *M* sodium lactate, 1 cc. phosphate buffer p_H 7·0, 1 cc. water and 1 cc. of the suspension of cells were placed in each Thunberg tube, and the tubes were evacuated and placed in a water-bath at 40°. At 30 and 60 minute intervals tubes were removed and the nitrite in them was estimated, the concentration of cells being adjusted so that after 30 minutes roughly 1 % of the nitrate was reduced. The two points almost always lay on a straight line through the origin, and experiments showing any significant departure from linearity were discarded.

The effect of cyanide on nitrate reduction.

Most biological oxidations involving the use of molecular oxygen are strongly inhibited (though not always completely) by low concentrations of cyanide of the order of 0·0001–0·001 *M*. In the case of *Bact. coli* it has been shown [Cook, Haldane and Mapson, 1931] that the inhibition of the oxidation of formate, lactate and succinate tends towards 100 % as the cyanide concentration is increased.

The inhibitory effect of cyanide on nitrate activation may be shown qualitatively in the following simple way. Four sets of vacuum tubes containing the following solutions

	Buffer cc.	Methylene blue cc.	KNO_3 *M*/40 cc.	KCN *M*/200 cc.	Water cc.	Bact. susp. cc.	Reduction time at 40° min.
1	1	1	—	—	2	1	7·75
2	1	1	1	—	1	1	∞
3	1	1	—	1	1	1	5·25
4	1	1	1	1	—	1	8

were evacuated and filled with hydrogen, with the usual precautions for preventing loss of hydrogen cyanide. The reduction times show that whilst nitrate prevents the reduction of methylene blue, this inhibition is almost completely removed by cyanide.

Quantitatively each of the two methods described has been applied to the measurement of the inhibition, with similar results. With the Barcroft manometer method special precautions must be taken to prevent loss of hydrogen cyanide, either in filling the cups with hydrogen or by absorption in the potash which usually occupies the small tubes inside the cups. The former difficulty was overcome by thoroughly evacuating the solutions used before placing them in the apparatus, to remove dissolved oxygen, and then partially evacuating and filling with hydrogen several times until the amount of air left was negligible; the latter by omitting the potash from the cups. This was justified by the fact that no carbon dioxide can be produced in the reaction and also by direct comparison of experiments with and without potash. In this way the velocity of hydrogen uptake at various concentrations of potassium cyanide was compared with that in the absence of cyanide.

The results with lactate as donator were obtained by using vacuum tubes with hollow stoppers to contain the cyanide solution, which was mixed with the rest of the contents of the tube after evacuation; the alkalinity of the potassium cyanide solution was sufficient to prevent appreciable loss of the acid during evacuation.

The results of all the measurements are given in Fig. 5. Toluene treatment

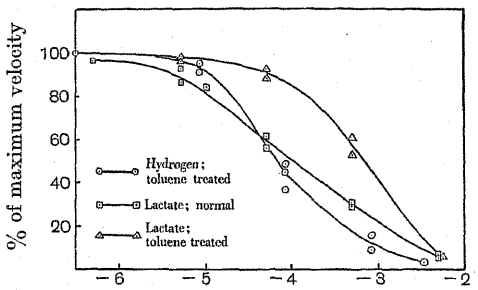


Fig. 5.

of the suspension has a considerable effect on the inhibition of nitrate reduction by cyanide, making the enzyme more susceptible to the reagent.

The extent of the inhibition does not depend on the nitrate concentration, *i.e.* it is as usual non-competitive.

KCN *M*	KNO_3 *M*	Velocity (*M* NO_2^-/hr.)	Velocity of control	Inhibition %
0·00002	0·02	0·000043	0·000114	62
0·00002	0·001	0·000035	0·000101	65

The effect of carbon monoxide.

The Barcroft manometer method of measuring the velocity of reduction cannot be applied in this case, as carbon monoxide has an inhibiting effect on hydrogenase which would interfere; the following figures show the effect of about half an atmosphere pressure of carbon monoxide on the rate of reduction of methylene blue by hydrogen in vacuum tubes with a toluene-treated suspension of *Bact. coli*:

Hydrogen mm.	Carbon monoxide mm.	Reduction time (mins.)
200	0	7·5
180	325	18·75

The lactic dehydrogenase is entirely unaffected by carbon monoxide [Cook, Haldane and Mapson, 1931], so the vacuum tube method can be applied; this is one of the reasons why lactate was chosen as the standard donator in this work.

Wherever carbon monoxide inhibits respiration, the inhibition is found to be a competitive one, so if the analogy between oxygen respiration and nitrate

respiration is to be borne out the inhibition by carbon monoxide should be greatest at the lowest nitrate concentrations. Neither at high or low nitrate concentrations was any inhibition observed, *e.g.*

KNO_3 (M)	CO (mm.)	Velocity (M NO_2^-/hr.)
0·02	0	0·000431
	117	0·000438
	325	0·000433
0·002	0	0·000384
	120	0·000410
	315	0·000391
0·0002	0	0·0000270
	650	0·0000265

It is essential in these measurements to find the initial velocity, as any change in the nitrate concentration would produce a double error, partly due to the assumed increase in inhibition by carbon monoxide at the lower nitrate concentration, and partly to the fact that the enzyme is not saturated with its substrate at 0·0002 M, so that the velocity would fall off as the concentration decreased. The lowest nitrite concentration that can be estimated with reasonable accuracy by the method employed here is 0·00001 M, so that if anything like an initial velocity is to be measured the initial concentration of nitrate must not be taken below, say, 0·00004 M.

Using this minimum concentration of nitrate, several experiments have been carried out. 3 cc. of each of 0·0002 M potassium nitrate, 0·1 M sodium lactate, phosphate buffer at p_H 7·0 and water were measured into each of six 100 cc. Büchner flasks, and 3 cc. of cell suspension added at such a dilution that about 0·00001 M nitrite would be expected in 30 minutes. The flasks were evacuated and one-half of them filled with carbon monoxide purified from oxygen. After 30 minutes' immersion in the water-bath, with vigorous shaking at first to ensure saturation with the gas, they were taken out and 10 cc. of their contents used for nitrite estimations. Typical results were

		Velocity (M NO_2^-/hr.)
1	Without CO	0·0000164
	With CO (about 1 atmosphere)	0·0000160
2	Without CO	0·0000212
	With CO (about 1 atmosphere)	0·0000203

These differences are not outside the experimental error, so the conclusion is that at a concentration of nitrate of 0·00004 M one atmosphere pressure of carbon monoxide (about 0·0007 M CO in solution) produces no measurable inhibition.

The effect of oxygen.

Before this subject could be investigated, a few preliminary results were required. First, it was shown that *Bact. coli* cannot re-oxidise nitrite to nitrate by means of oxygen. Tubes containing buffered 0·00025 M sodium nitrite and a thick cell suspension were aerated with a current of air saturated with water vapour, and samples were withdrawn for estimation of nitrite. No re-oxidation was observed.

Time (mins.)	$NaNO_2$ (M)
0	0·000246
30	0·000248
60	0·000260
140	0·000247

Second, the rates of reduction of nitrate in the absence of oxygen and of oxygen in the absence of nitrate were compared directly. It was found that, taking one atom of oxygen as comparable with one molecule of nitrate, oxygen is reduced about ten times as fast as nitrate, *e.g.*

Normal suspension	0·0221 g. atoms O_2 per hour 0·00236 g. mols. KNO_3 per hour
Toluene-treated suspension	0·0034 g. atoms O_2 per hour 0·000352 g. mols. KNO_3 per hour

To investigate the inhibition of nitrate reduction by oxygen quantitatively, it was necessary to vary the concentration of oxygen used, and this was done by diluting air with nitrogen and bubbling the mixtures through the solutions. The details of the method employed are as follows. A large aspirator was filled with the gases in roughly the right proportions, and the oxygen in the mixture estimated. 2 cc. of 0·1 M sodium lactate, phosphate buffer at p_H 7·0, water and nitrate solution were measured into each of a series of large test-tubes with corks, connected with pressure tubing, and a rapid stream of the gas bubbled through for about 15 minutes to saturate the solutions with oxygen at the right pressure. Then 2 cc. of the suspension of bacteria were added, and the bubbling was continued while samples were withdrawn for estimation of nitrate at various intervals. The velocity of nitrate reduction *in vacuo* was determined simultaneously. To make sure that at the lowest oxygen pressures no appreciable part of the oxygen was being removed from the gas, a sample was collected in one experiment as it issued from the last tube, and analysed. Before passing through the tubes the gas gave duplicate analyses of 0·42 % and 0·41 % oxygen, and after, 0·41 % and 0·44 %.

A series of results was obtained with nitrate concentrations of 0·02 M and 0·002 M; the extent of the inhibition was identical in the two cases, so only one set of figures (those for 0·02 M) is given. These results are plotted in Fig. 6, the concentrations of oxygen being given logarithmically.

O_2 %	O_2 in sol. M	Velocity	Velocity of control	Inhibition %
99	0·00100	0·000046	0·00120	96
20·9	0·00021	0·000026	0·000513	94
3·76	0·000038	0·000026	0·000387	93
1·65	0·000017	0·000047	0·000412	88
1·09	0·000011	0·000136	0·000352	61
0·42	0·0000042	0·000259	0·000350	26
0·36	0·0000036	0·000446	0·000568	21

For reasons which will be explained in the discussion of these results, the effect of carbon monoxide on the inhibition of nitrate reduction by oxygen was investigated. Experiments similar to those already described were done

with mixtures containing different amounts of oxygen with roughly ten times as much carbon monoxide. The inhibition was in each case much decreased (see Fig. 6).

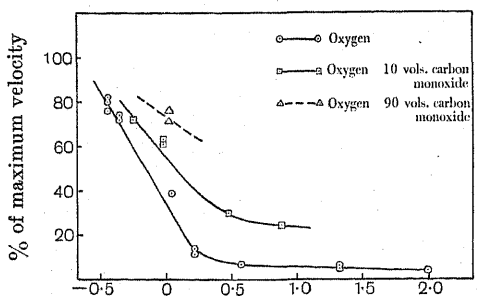


Fig. 6.

O_2 %	CO %	CO/O_2	O_2 in sol. M	CO in sol. M	Velocity	Velocity of control	Inhibition %
7·62	84·3	11·1	0·000077	0·00063	0·000124	0·000508	75·5
3·00	31·9	10·6	0·000030	0·00024	0·000132	0·000440	70
0·94	9·8	10·5	0·0000095	0·000073	0·000227	0·000370	39
0·55	4·5	8·9	0·0000056	0·000033	0·000398	0·000528	24
In addition one experiment was done with a larger CO/O_2 ratio:							
1·03	91·2	89	0·0000104	0·00068	0·000296	0·000391	24

A further question of importance is whether the extent of inhibition by oxygen depends on the concentration of nitrate. As already stated, initial concentrations of nitrate of 0·02 *M* and 0·002 *M* gave identical results, in the presence or absence of carbon monoxide, at all concentrations of oxygen between 0·000004 *M* and 0·001 *M* (0·4 % and 99 % of an atmosphere). The following results, obtained in the same way, show that the inhibition is also the same with 0·0002 *M* nitrate, a concentration at which the velocity of reduction is already falling off owing to unsaturation of the enzyme.

O_2 %	O_2 in sol. *M*	KNO_3 *M*	Velocity	Velocity of control	Inhibition %
0·36	0·0000036	0·02	0·000446	0·000568	21
,,	,,	0·002	0·000416	0·000547	24
,,	,,	0·0002	0·000332	0·000402	18

A few experiments were carried out to see if nitrate affects the velocity of reduction of oxygen. Two pairs of Barcroft manometers were set up, one pair with 0·02 *M* sodium lactate, the other with 0·02 *M* lactate and 0·01 *M* nitrate, both in air. The nitrate seemed to slow down the oxygen uptake about 5 %, but this is not really outside the experimental error, and cannot be considered significant.

Discussion.

There is no difficulty in deciding between the three possibilities mentioned in the introduction, with regard to the enzymes concerned in the activation of oxygen and of nitrate.

First, the relative affinities of the enzymes for their substrates and for carbon monoxide show that two entirely different mechanisms are concerned. Cook, Haldane and Mapson [1931] state that oxidations by oxygen are still working at their maximum velocity in oxygen at 4 % of an atmosphere pressure, *i.e.* at 0·00004 *M*, so that the Michaelis constant of the oxygen enzyme for its substrate is at any rate less than 0·000004 *M*; and they showed that the affinity of the enzyme for carbon monoxide is one-tenth that of its affinity for oxygen (when lactate is the hydrogen donator), so that the Michaelis constant of carbon monoxide for the oxygen enzyme is at any rate less than 0·00004 *M*. In the case of nitrate, the Michaelis constant for the substrate is about 0·00005 *M*, while carbon monoxide (0·0007 *M*) causes no inhibition in the presence of 0·00004 *M* nitrate, so that the Michaelis constant of the nitrate enzyme for carbon monoxide must be at any rate greater than 0·007 *M*. The values $< 0{\cdot}00004$ and $> 0{\cdot}007$ are sufficiently divergent to show that the two enzymes cannot be the same.

Second, if the same enzyme activated both oxygen and nitrate, the inhibition of the reduction of one substrate caused by the presence of the other should be competitive, whereas the results show that the amount of inhibition at any oxygen pressure is independent of the nitrate concentration. Thus the facts can be accounted for only by assuming two separate specific enzymes.

There remains to be explained the fact that oxygen causes a non-competitive inhibition of nitrate reduction, and a simple and qualitatively satisfactory explanation will now be given. Lactic dehydrogenase is providing a constant supply of activated lactic acid, and this, or the intracellular hydrogen carriers reduced by it, is shared between the oxygen and the nitrate enzymes. When both these enzymes are saturated with their substrates, the relative amounts reduced will depend on the "concentrations" of the enzymes and on various other factors. Considering the facts that oxygen is reduced ten times as fast as nitrate, and that the maximum inhibition of nitrate reduction by oxygen is 96 %, it will be seen that under these conditions 0·4 % of the available lactate is oxidised by nitrate and 99·6 % by oxygen. If now the rate of activation of oxygen is decreased by lowering the oxygen pressure, a greater proportion of the lactate will be oxidised at the nitrate enzyme; *e.g.* at 0·36 % oxygen, giving 20 % inhibition of the nitrate reduction, 8 % of the available lactate is oxidised by nitrate, 92 % by oxygen. Similarly, if the activation of oxygen is inhibited by carbon monoxide, a greater proportion of nitrate will be reduced; a CO/O_2 ratio of ten, giving 50 % inhibition of oxygen uptake, reduced the limiting inhibition from 96 % to about 80 %, so that 2 % of the lactate was oxidised by nitrate and 98 % by oxygen. It can be seen that nitrate cannot alter the rate of oxygen uptake sufficiently to give significant results on a Barcroft manometer apparatus.

Though the system is too complicated for exact mathematical treatment, the facts are qualitatively explained by this view.

In its behaviour with cyanide the nitrate-activating enzyme is similar to

other oxidising enzymes such as indophenol oxidase and peroxidase; with *Bact. coli*, using lactate as hydrogen donator, 50 % inhibition of the oxygen-activating enzyme is produced by 0·001 *M* cyanide, and 50 % inhibition of the nitrate enzyme by 0·0001 *M*. The inhibition is non-competitive in both cases [Cook, Haldane and Mapson, 1931]. The lack of inhibition by carbon monoxide is not unusual; Dixon [1928] has shown that the catalysis of the oxidation of cysteine by haematin is not inhibited by this reagent.

Summary.

1. *Bact. coli* reduces nitrate quantitatively to nitrite. After toluene treatment it oxidises formate, lactate and succinate to the same degree by means of nitrate as it does with oxygen, *i.e.* to carbon dioxide, pyruvate and fumarate respectively.

2. The reduction can be completely inhibited by cyanide, 50 % inhibition being produced by about 0·0001 *M* KCN.

3. As far as the method used allowed, no inhibition by carbon monoxide could be observed.

4. Oxygen causes a non-competitive inhibition of nitrate reduction; this inhibition is partly removed by carbon monoxide.

5. A theory is offered which explains these results qualitatively.

I wish to record my thanks to Miss Stephenson, Drs Dixon and Woolf, and Prof. J. B. S. Haldane for useful advice, and to Sir F. G. Hopkins for much help and encouragement.

References.

Cook (1930). *Biochem. J.* **24**, 1538.
—— Haldane and Mapson (1931). *Biochem. J.* **25**, 534.
—— and Stephenson (1928). *Biochem. J.* **22**, 1368.
Dixon (1928). *Biochem. J.* **22**, 902.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
—— and Whetham (1924). *Biochem. J.* **18**, 519.
—— and Wooldridge (1927). *Biochem. J.* **21**, 148.
Stephenson and Stickland (1931). *Biochem. J.* **25**, 205.

CLXIX. STRUCTURE AND ENZYME REACTIONS.

X. ACTION OF SALTS ON THE SYSTEMS: AMYLASE-STARCH-PROTEINS.

By HEDWIGA CHREMPIŃSKA.

From the Biochemical Laboratory, Faculty of Medicine, Warsaw University.

(*Received August 7th, 1931.*)

In the present paper the results obtained from a study of the effect of addition of electrolytes (NaCl, $MgCl_2$, $CaCl_2$) to systems containing malt-amylase, starch, and gelatin, ovalbumin, or egg-white will be described.

Experimental.

Methods.

Solutions of Merck's starch were prepared by boiling with water. The amylase used was Merck's "Diastase absolut." Solutions not containing protein were buffered either by Sørensen's phosphate solutions or by glycine-sodium chloride-sodium hydroxide solutions. p_H was determined potentiometrically, using a calomel electrode. Gelatin purchased from Coignet Père et Fils (Paris) was used, and Merck's ovalbumin, solutions of which were prepared by dissolving in water, leaving 12 hours and filtering. The p_H of protein solutions was adjusted by addition of the requisite quantities of 0·1 *N* NaOH or HCl. The salts used were Merck's analytical reagents. The experiments were in most cases performed at 20°, and the velocity of reaction was determined either from the final reducing sugar content, determined by Bertrand's method, or from the final starch content, determined by Pflüger's glycogen method.

Results.

I. *Effect of salts in the absence of proteins.*

Action of sodium chloride. This was studied at p_H 3·6 and 5·2, *i.e.* above and below the optimal p_H 4·5. The results (Table I) show that at p_H 5·2 no effect is observed in concentrations of less than *N*; in 2*N* solutions of NaCl hydrolysis amounts to 53 % and in 3*N* solutions to 8 % of the normal value. At p_H 3·6 the inhibitory action of sodium chloride appears at lower concentrations, hydrolysis being 88·3 % in 0·1 *N*, 63 % in 0·2 *N* and 40·7 % in 0·3 *N* NaCl.

Table I. *Action of amylase in presence of NaCl.*

Systems contain 10 cc. Sørensen's phosphate buffers, 10 cc. 1·5 % starch, 10 cc. NaCl solution, and 1 cc. 0·1 % amylase. Temp. 20°. Duration of reaction: 4 hours for systems at p_H 5·2, 5 hours for systems at p_H 3·6. Maximum yield of maltose 120 mg.

Conc. NaCl *N*	Maltose produced: At p_H 3·6 mg.	% of yield without NaCl	At p_H 5·2 mg.	% of yield without NaCl
0	36·5	100	77·9	100
0·02	35·9	—	77·4	99·4
0·05	34·2	—	83·1	
0·10	30·2	88·3	83·6	
0·20	21·4	63·0	83·4	
0·30	13·9	40·7	83·8	107
0·60	—	—	84·4	
0·80	—	—	84·0	
1·0	—	—	83·7	
2·0	—	—	41·3	53
3·0	—	—	6·1	8
4·0	—	—	6·3	8

Action of calcium chloride. Similar results were obtained with calcium chloride, in solutions buffered to p_H 3·6, 5·2 and 6·8 (Table II). The velocity of reaction fell at p_H 3·6 to 90 % of normal in 0·072 *N* $CaCl_2$, 51·8 % in 0·144 *N*, 8 % in 0·2–0·9 *N*, and 4·2 % in 1·1 *N* salt. At p_H 5·2 the inactivating effect was observed first in 0·444 *N* solutions of $CaCl_2$, in which the velocity of hydrolysis was diminished by 18 %, and further increase in concentration of $CaCl_2$ led to a further slow decline. At p_H 6·8 three periods were observed: in the first, the addition of $CaCl_2$ enhanced hydrolysis by about 40 %, this optimal value being maintained over a range of 0·018–0·222 *N* $CaCl_2$, whilst in higher concentrations the velocity of hydrolysis again fell. Thus in 0·444 *N* solutions it amounted to 90 % of the original value, in 0·666 *N* $CaCl_2$ to 32 %, and in 0·888 *N* solutions to 1·6 %. At both p_H 5·2 and 6·8 the inhibitory action of $CaCl_2$ was first manifested in 0·444 *N* solutions.

Table II. *Effect of $CaCl_2$.*

Systems contain 15 cc. of 1·5 % starch, 1 cc. 0·1 % amylase without NaCl, and 5 cc. $CaCl_2$ or H_2O. The control systems yielded 37·7 mg. glucose on hydrolysis.

Conc. $CaCl_2$ *N*	Starch decomposed (expressed as glucose): At p_H 3·6 mg.	% of yield without $CaCl_2$	At p_H 5·2 mg.	% of yield without $CaCl_2$	At p_H 6·8 mg.	% of yield without $CaCl_2$
0	16·6	100	17·7		8·6	100
0·018	17·4	106	18·2		12·7	147·6
0·086	17·9		17·7	100	12·4	144·2
0·072	14·9	90·0	18·5		12·6	146·5
0·144	8·6	51·8	17·7		12·4	144·2
0·222	1·4		17·9		13·2	153·5
0·444	1·4	8·4	14·5	82·0	11·4	132·0
0·666	2·6		8·1	46·0	4·1	47·9
0·888	1·4		4·7	26·6	0·2	1·6
1·100	0·7	4·2	4·8	26·8	0·2	

Action of aluminium chloride. The action of this salt was studied at p_H 5·2, and the solution of $AlCl_3$ was previously reduced to this p_H by addition of

0·1 *N* NaOH. The velocity of amylolysis was reduced to 65·3 % of the original value by 0·025 *N* $AlCl_3$, and to 44·4 % by 0·05 *N* aluminium chloride (Table III).

Table III. *Effect of* $AlCl_3$.

Systems contain 10 cc. 1·8 % starch, 15 cc. $AlCl_3$ or H_2O, 1 cc. 0·1 % amylase (without NaCl), and 6·7 cc. H_2O or 0·1 *N* NaOH for neutralisation of $AlCl_3$. Systems kept 4 hours at 20° p_H 5·2.

Conc. $AlCl_3$ *N*	Maltose produced mg.	% of yield without $AlCl_3$
0	34·0	100
0·025	22·2	65·3
0·050	15·1	44·4
0·100	11·6	34·1
0·200	5·1	15·0

II. *Action of salts in systems containing gelatin.*

The systems contained 0·6–1·2 % gelatin, warm solutions of which were added to starch solutions with or without electrolyte, and the mixtures were left for 12 hours, after which 1 cc. of 0·01 % amylase in 0·1 % NaCl was added with stirring to the semi-gelated mass. All systems were at p_H 5·7. Systems containing sodium chloride exhibited a feeble Tyndall effect; this was much more evident in the presence of calcium chloride.

Action of sodium chloride. The results obtained (Table IV) indicate that sodium chloride has, after a certain concentration, an activating effect, 15 % more maltose being produced in 0·02 *N*, and 23 % more in 0·04 *N* NaCl than in the absence of this salt.

In view of Freiberger's observation [1931] that the apparent reducing sugar content of solutions as determined by Bertrand's method is smaller in the presence of gelatin than in its absence, experiments were performed on solutions containing maltose and gelatin, with and without salts. The values found were the same in both cases, indicating that the activation found is not due to methodical errors, but to an actual increase in the activity of amylase.

Table IV. *Action of NaCl in systems containing gelatin.*

10 cc. 0·6 % starch, 10 cc. 1·2 % gelatin, 1 cc. 0·01 % amylase in 0·1 % NaCl, and 2 cc. of salt solution or H_2O. Systems kept 4 hours at 20° (p_H 5·7). Maximum yield of maltose 45 mg.

	Maltose produced			
	Exp. 1		Exp. 2	
Conc. NaCl *N*	mg.	% of yield without NaCl	mg.	% of yield without NaCl
0·0	9·0	100	7·3	
0·002	8·6	95·5	7·6	
0·004	8·6	95·5	7·3	100
0·008	8·7	96·7	6·9	
0·012	8·7	96·7	7·2	
0·016	9·3	103·3	7·4	
0·020	11·3	126·6	8·4	115
0·032	11·4	126·7	9·0	123·3
0·040	11·4	126·7	9·0	123·3

Action of potassium chloride. The results obtained with KCl are identical with those found for NaCl.

Action of calcium chloride. Calcium chloride at certain concentrations diminishes velocity of reaction at 18°, but not at 30°. The percentage retardation varies from 5 to 23 % in the initial stages of the reaction; given sufficient time, the reaction proceeds in all cases to completion. The addition of potassium chloride to systems containing calcium chloride diminishes the inactivating effect of the latter salt.

Table V. *Action of $CaCl_2$ in systems containing gelatin.*

10 cc. 0·6 % starch, 10 cc. 1·2 % gelatin, 1 cc. 0·01 % amylase in 0·1 % NaCl, and 2 cc. of salt solution or H_2O. Systems kept 4 hours at 20°; p_H 5·7.

		Starch decomposed		
No. of exp.	Time of reaction (hours)	Without $CaCl_2$ mg.	In presence of 0·032 *N* $CaCl_2$ mg.	% retardation
1	1	15·0	11·6	22·6
	2	40·4	35·7	11·6
2	0·5	4·4	3·8	13·6
	1	5·5	4·4	20·0
3	0·5	8·7	7·0	19·5
	1·0	15·1	12·9	14·6
	12·0	45	45	0
4	0·5	6·1	5·8	5·0
	1	11·0	11·0	9·1
	24	44·8	45·1	0

III. *Action of salts in systems containing ovalbumin.*

Amylase was added to the following systems: (*a*) 5 cc. 1 % starch solution and 5 cc. of about 4 % Merck's ovalbumin; (*b*) the same, boiled; (*c*) ovalbumin solution first boiled, and starch solution then added; (*d*) starch solution added

Table VI. *Action of dissolved and heat-coagulated ovalbumin in presence and absence of $CaCl_2$.*

The starch present was equivalent to 42·4 mg. glucose.

	Starch hydrolysed (mg.) at			
	p_H 5·2			p_H 6·1
	0·01 % amylase		0·1 % amylase	0·01 % amylase
System	1 hour	2 hours	4 hours	4 hours
(*a*) Ovalbumin + starch + amylase	11·2	19·7	35·7	8·2
(*b*) Ovalbumin + starch, heated at 100°, then amylase	12·7	20·1	35·5	8·2
(*c*) Ovalbumin heated at 100°, then starch and amylase	11·2	19·7	35·3	8·3
(*d*) Ovalbumin + starch + $CaCl_2$ + amylase	12·7	20·1	35·5	8·7
(*e*) Ovalbumin + starch + $CaCl_2$ heated at 100°, then amylase	11·2	20·1	35·5	8·3
(*f*) Ovalbumin + $CaCl_2$, heated at 100°, then starch and amylase	11·2	20·1	35·3	8·7

to ovalbumin solution containing $CaCl_2$; (*e*) the same, boiled, and (*f*) starch added to boiled ovalbumin-$CaCl_2$ solution. The experiments were carried out at p_H 5·2 and 6·06. 1 cc. of 0·2, 0·1 or 0·01 % amylase was added to all systems, and 1 cc. of water or of 1·2 *N* $CaCl_2$ solution. The results (Table VI) indicate that the velocity of hydrolysis is independent of the state of the ovalbumin (sol or gel) and of the presence of $CaCl_2$ (0·1 *N*).

IV. *Action of salts in systems containing egg-white.*

Coagulated egg-white. 1 cc. of water or $CaCl_2$ in various concentrations and 10 cc. of 1 % starch solution were added to 5 g. of heat-coagulated egg-white and the systems were shaken for 1 hour, after which 1 cc. of 0·1 % amylase in 0·1 % NaCl solution was added, and residual starch was determined after 5 hours by Pflüger's method. The results (Table VII) indicate that 0·01 *N* $CaCl_2$ accelerates hydrolysis by 5·2 %, and 0·2 *N* $CaCl_2$ by 32 %. Considerably greater activation was observed when the time of shaking before addition of amylase was prolonged to 4 hours, when the acceleration of reaction due to 0·1 *N* $CaCl_2$ was not 24 %, as after 1 hour's shaking, but 55 %, whilst in 0·2 *N* $CaCl_2$ it was now 60 %. The activating effect of the addition of calcium chloride was evident in concentrations at which no effect was observed in the absence of protein.

Table VII. *Action of $CaCl_2$ in systems containing egg-white.*

Starch present equivalent to 74·0 mg. glucose. Residual starch determined by Pflüger's method.

	Shaken 1 hour		Shaken 4 hours	
Conc. $CaCl_2$ *N*	Starch decomposed mg.	% acceleration due to $CaCl_2$	Starch decomposed mg.	% acceleration due to $CaCl_2$
0	23·2	—	27·0	—
0·01	24·4	5·2	—	—
0·02	24·6	5·3	—	—
0·05	27·3	18	34·7	28·5
0·10	28·8	24	41·5	54·0
0·20	30·6	32	44·2	64·0

The p_H of the systems was originally 8·8, but fell to 8·1 on addition of calcium chloride; this change in p_H leads to an acceleration of reaction amounting to about 43 %.

Sodium chloride (0·1 *N*) has no influence on the velocity of hydrolysis in analogous systems. It was, however, observed that the addition of NaCl to systems containing $CaCl_2$ diminished the activation due to the latter salt. Thus we see (Table VII) that in the presence of 0·1 *N* NaCl the acceleration due to 0·1 *N* $CaCl_2$ was only 23 %, whilst with 0·05 *N* $CaCl_2$ no acceleration was observed.

The addition of sodium sulphate (0·05–0·2 *N*) has no influence on the system starch-amylase-coagulated egg-white (Table VIII).

Comparison of the action of dissolved and coagulated egg-white. 5 cc. of egg-white were added to 5 cc. of water, 10 cc. of 1 % starch, 1 cc. amylase in

0·1 % NaCl, and 1 cc. of water or 2·2 *N* $CaCl_2$ were added. Egg-white was coagulated in certain of the systems by heating for 2 minutes at 100°, in the presence or absence of $CaCl_2$, and amylase was added to the cooled systems. The results (Table IX) show that in the absence of electrolytes the coagulation of egg-white reduces the velocity of reaction by 41–54 %. $CaCl_2$ in concentrations up to 0·1 *N* accelerates reaction both in the presence of dissolved and of coagulated egg-white; this acceleration amounted respectively to 40–56 % and 86–142 %, the velocity of reaction in the presence of coagulated egg-white and $CaCl_2$ being greater than in the presence of dissolved egg-white without $CaCl_2$.

Table VIII. *Systems as in Table VII, but different salts.*

Salt	Starch decomposed (expressed as mg. glucose)
—	30·4
0·1 *N* NaCl	28·9
0·1 *N* NaCl + 0·05 *N* $CaCl_2$	29·7
0·1 *N* NaCl + 0·1 *N* $CaCl_2$	37·3
—	30·8
0·05 *N* Na_2SO_4	30·8
0·10 *N* Na_2SO_4	30·2
0·20 *N* Na_2SO_4	30·2

Table IX.

Duration of reaction 4 hours; p_H without $CaCl_2$ 8·8, with $CaCl_2$ 8·1.

System	Starch decomposed mg.	% decomposition as compared with system (*a*)
(*a*) Dissolved egg-white + starch	29·2–31·5	100
(*b*) „ „ „ + $CaCl_2$	42·5–46·1	135–158
(*c*) Egg-white + starch, heated at 100°	14·1–17·0	44·8–58·6
(*d*) Egg-white + starch + $CaCl_2$, heated at 100°	31·7–34·8	109–111

Discussion.

The action of salts in systems containing amylase and starch is quantitatively different, according to whether the systems are above or below the optimal p_H of the amylase; in the former case the same effect is achieved by 10 times higher concentrations than in the latter. At p_H 7 $CaCl_2$ accelerates reaction when present in small concentrations. At p_H 5·2 the action of salts appears to be a function of the valency of the cation; thus the minimal retarding normal concentrations of the salts $AlCl_3$: $CaCl_2$: NaCl are as 1 : 18 : 60. At p_H 3·6 this dependence is not observed, the actions of the salts being roughly equal at equivalent concentrations. At p_H 5·2 and 3·6 no acceleration of reaction was observed in the presence of low concentrations of salts, and this may be ascribed to the fact that the enzyme preparation was not previously freed from salts by dialysis, and to the relative insensitiveness of vegetable amylase to mineral salts [Sherman and Tauberg, 1916; Saito, 1906]. The action of salts is very different according to the variety of protein present, and to its physical state. In systems containing gelatin, the addition of

sodium chloride leads to acceleration of reaction (23 % acceleration in 0·04 *N* solution). The causes of this effect are not clear; it may be due to combination with gelatin of the NaCl added with the amylase solution, and necessary for its action, and hence the addition of further NaCl accelerates reaction. Experiments with $CaCl_2$, however, do not support this hypothesis, but suggest that the action of NaCl is rather in the direction of affecting the degree of aggregation of gelatin, thereby facilitating the penetration of amylase into protein aggregates containing occluded starch.

Calcium chloride exerts a retarding action, in contradistinction to NaCl. Since the retarding concentrations of calcium chloride in such systems are considerably smaller than the smallest active concentrations in systems not containing protein, the explanation of its action should be connected with the presence of protein, and this view is further supported by the fact that at 30° no retardation due to the presence of $CaCl_2$ can be observed, and that, given sufficient time, the final yield of maltose is the same in the presence or absence of $CaCl_2$. Observations of the Tyndall effect and of viscosity in jellified systems indicate that $CaCl_2$ enhances the compactness of the jelly (Targońska, unpublished work), and the possibility may therefore exist that the penetration of amylase within starch-containing aggregates is rendered more difficult in the presence of this salt.

The results of experiments conducted on systems containing ovalbumin or egg-white are even more difficult of interpretation than are those for gelatin. Filipowicz (unpublished work) has found that the velocity of reaction of amylase with starch is accelerated by the addition of proteins, in systems having p_H values considerably higher than the optimal for amylase; this acceleration is considerable, and may under suitable conditions amount to over 40 %. A second factor influencing the velocity of reaction is the change in p_H due to addition of neutral salts; this, as has been shown by Przyłęcki and Giedroyć [1931], may be quite considerable in alkaline systems in the presence of protein. Filipowicz has, however, found that changes in p_H have a much feebler effect on the activity of amylase in systems containing protein than in its absence. In this case, we see (Table X) that 0·1 *N* $CaCl_2$ reduced the p_H of a system containing protein and starch from 8·8 to 8·1, and that this change in p_H itself leads to an acceleration of reaction of 43·3 %.

Table X.

Systems contain 5 cc. egg-white, 5 cc. water, 10 cc. 1 % starch, 1 cc. 0·1 % amylase, and 1 cc. of $CaCl_2$, HCl, or water.

System	p_H	Starch decomposed mg.	% acceleration
As above, with water	8·8	33·3	—
,, HCl	8·1	47·7	43·3
,, $CaCl_2$	8·1	50·0	50·1

As to possible changes produced by salts on the state of combination (this we shall term "sorption," without entering into the question of the chemical,

physical, or mechanical nature of the forces to which the phenomenon is due) of starch or amylase with protein, our knowledge is very inadequate, at least in so far as systems containing protein sols are concerned. Both NaCl and $CaCl_2$ diminish sorption, to a greater extent when added before coagulation of the sol than when added after. The reversal of the process of sorption, where it has already taken place, requires considerably higher concentrations of sodium chloride than of calcium chloride, and the process of desorption proceeds very slowly in both cases, equilibrium being attained in about 4 hours. In systems to which $CaCl_2$ has been added after coagulation of protein 64 % of the total starch remains in solution after shaking for 1 hour, whilst in the absence of $CaCl_2$ only 55 % remains, a difference of 17 %. After a further 3 hours, the corresponding figures are 51 and 27 %, and the difference now amounts to 51 %. When $CaCl_2$ (0·1 N) is added before coagulation of protein, 55 % of the total starch remains in solution, as compared with 20 % in the absence of $CaCl_2$, or 175 % more. Sodium chloride, in the concentrations taken (up to 0·05 N), had very little influence on the proportion of starch remaining in solution (22 % with NaCl, 15 % without).

Coagulated ovalbumin combines with starch to a much smaller extent than does coagulated egg-white. Neither dissolved nor coagulated ovalbumin retards the velocity of amylolysis, either in the presence or absence of $CaCl_2$, whence it appears that in such systems the reduction in concentration of the substrate in solution is without effect on the velocity of reaction, in spite of the fact that in homogeneous systems the latter is reduced by about 50 % by a similar change in concentration. The velocity of reaction is, further, independent of whether the ovalbumin was coagulated in the presence or the absence of starch. Occluded starch is liberated in alkaline solution in the same way as sorbed starch, in contradistinction to acid systems (p_H 3), in which both occlusion and sorption are irreversible [Przyłęcki and Gurfinkel, 1930; Przyłęcki and Majmin, unpublished work].

In systems containing egg-white quite different results were obtained, the velocity of reaction being considerably smaller in the presence of coagulated than of soluble protein. This relative retardation amounts in the systems studied to about 50 %, whilst at the same time sorption amounts to about 80 %. The disproportionality between these figures may be ascribed to the partial combination of starch or enzyme with protein sol; alternative explanations would be that it is due to the greater viscosity of systems containing soluble proteins, or that the reason for the smaller velocity observed in the presence of coagulated egg-white lies not in sorption but in change in concentration of activating protein. The effect of addition of $CaCl_2$, however, supports the view that sorption is the cause of retardation of reaction, as this salt accelerates reaction in the presence of dissolved and coagulated egg-white respectively by 50 and above 100 %.

This activation may be due largely to reduction in p_H of unboiled systems, in which a change from p_H 8·8 to 8·1 accelerates reaction by 43 %; this effect

does not, however, apply equally to systems containing coagulated egg-white. Here the following facts support the view that $CaCl_2$ acts by desorption, with consequent increase in active concentration. The same concentration of $CaCl_2$ has more than twice as great an effect in systems containing coagulated than dissolved egg-white. The percentage acceleration of reaction is more than doubled by shaking systems containing coagulated egg-white and $CaCl_2$ for 4 hours instead of 1 hour, whilst where egg-white is coagulated in the presence of $CaCl_2$ it is about four times as great. These changes in the velocity of reaction vary roughly in parallel with the variations in the proportion of dissolved to sorbed starch in the above systems. In systems containing soluble egg-white, $CaCl_2$ may, apart from its usual effect of lowering the p_H of the systems, diminish the proportion of starch combined with protein particles. No obvious explanation is forthcoming as to the reason why the velocity of reaction in the presence of coagulated egg-white and $CaCl_2$ is greater than in systems containing dissolved egg-white without $CaCl_2$. It is possible that certain of the effects observed are due to variations in the active concentration of enzyme due to its adsorption on dissolved or coagulated protein; work on this aspect of the question is now being conducted.

The above observations may possess certain biological applications, chiefly in plant physiology. It is evident that vegetable cells possess agents, such as $CaCl_2$, which are able to affect the quantitative relation between free and sorbed starch in the cytoplasm, and that such influences may determine the velocity of enzymic hydrolysis; in systems in which the reaction of synthesis is in equilibrium with that of hydrolysis the presence of mineral salts may even determine the direction of reaction.

Summary.

1. Electrolytes retard the velocity of enzymic hydrolysis of starch. Systems of p_H below the optimal are more sensitive to the action of salts than are those of p_H higher than the optimal; in the latter case the active concentration of ions is a function of their valency.

2. The velocity of enzymic hydrolysis in systems containing gelatin is accelerated by the addition of NaCl, and retarded by the addition of $CaCl_2$.

3. In systems containing Merck's ovalbumin neither heat-coagulation nor $CaCl_2$ affects the velocity of reaction.

4. In systems containing crude egg-white heat-coagulation in the presence of $CaCl_2$ accelerates reaction. The velocity depends on the time during which the system containing coagulated egg-white and $CaCl_2$ is shaken before addition of amylase. NaCl and Na_2SO_4 are without effect in this system. In the absence of $CaCl_2$ heat-coagulation reduces velocity of reaction by 50 %.

5. The velocity of reaction in the presence of dissolved egg-white is increased by about 50 % by addition of $CaCl_2$. This effect is due to reduction

of p_H, as the same acceleration is obtained by addition of the appropriate quantity of HCl.

6. In systems containing heat-coagulated egg-white and $CaCl_2$, the acceleration observed is due probably to desorption of starch from protein.

REFERENCES.

Freiberger (1931). *Biochem. J.* **25**, 705.
Przyłęcki and Gurfinkel (1930). *Biochem. J.* **24**, 179.
—— and Giedroyć (1931). *Biochem. J.* **25**, 465.
Sherman and Tauberg (1916). *J. Amer. Chem. Soc.* **38**, 1638.
Saito (1906). *Zentr. Bakt. Par.* II, **17**, 20, 101, 152.

CLXX. THE OXIDATION OF SULPHYDRYL COMPOUNDS BY HYDROGEN PEROXIDE.

I. CATALYSIS OF OXIDATION OF CYSTEINE AND GLUTATHIONE BY IRON AND COPPER.

By NORMAN WINGATE PIRIE.

From the Biochemical Laboratory, Cambridge.

(*Received August 7th, 1931.*)

The oxidation of thiol compounds by oxygen and methylene blue has, in recent years, been very extensively studied, while their oxidation by hydrogen peroxide has suffered almost complete neglect. Abderhalden and Wertheimer [1923] found that cysteine, like other mercaptans, was instantaneously oxidised by hydrogen peroxide in neutral solution, and that this oxidation was unaffected by cyanide. At neutrality an oxidation of the thiol group would be complicated, at any rate in the presence of iron, by the Fenton reaction. Quastel, Stewart and Tunnicliffe [1923] have indeed prepared an oxidation product of this type from glutathione. In acid solution and in the presence of the theoretical amount of hydrogen peroxide the reaction proceeds smoothly with the production of pure oxidised glutathione [Pirie, 1931].

In this paper the results of a study of the oxidation of cysteine and glutathione to the corresponding disulphides will be given while in a later one a more deep-seated oxidation will be described. The former action is catalysed by iron, copper and thiocarbamide while the latter is catalysed by vanadium, titanium and molybdenum. The following elements have, in acid solution, either no action or a very slight one: Ag, As, Be, Co, Cr, Hg, Mn, Ni, Pb, U, W, Zn.

Experimental.

The course of the oxidation was followed polarimetrically since the ordinary methods which have been used for following oxidations are clearly inapplicable. Polarimeter tubes were made of heavy-walled glass tubing, 4 mm. internal diameter and 20 cm. long, with a side-tube through which they were filled. The ends (end plates for the ordinary type of polarimeter tube) were fixed on with de Khotinsky cement. Except for the part over the bore of the tube the end plates were blackened. When not in use the tubes, and all the other vessels used in this work, were kept filled with 20 % hydrochloric acid. Before use they were rinsed out five or six times with glass-distilled water and dried by connecting to a mechanical pump having an efficient sulphuric acid absorption tube.

"Metal-free" cysteine hydrochloride was prepared according to Dixon's method by recrystallisation four times from metal-free 20 % hydrochloric acid; metal-free glutathione was obtained by the method already described [Pirie, 1930] the vessels used for washing and decomposing the cuprous compound being washed with acid before use. Merck's "perhydrol" has a very low metal content and was used in all cases diluted to give a 0·44 *N* solution. At this concentration it is stable for some days but if diluted much further it tends to decompose.

Glass apparatus was used throughout, and, presumably because all the fluids used were acid, no trouble was experienced through metals being adsorbed by the glass.

The oxidation of cysteine.

In the absence of added metal the rate of oxidation of cysteine is very small at p_H 2·1 (the p_H of a dilute solution of cysteine hydrochloride) but is markedly increased by small amounts of iron or copper. These results are shown in Fig. 1. In each case the appropriate amount of a dilute solution of ferrous sulphate (freshly prepared each day) or copper sulphate was added to 1 cc. of a 1 % solution of cysteine hydrochloride, the amount of metal-free water necessary to make up the volume to 4·5 cc. was run in from a burette and finally 0·5 cc. of 0·44 *N* H_2O_2 was added. The mixture was then poured into a dry polarimeter tube and the rotation measured for mercury green light. It is clear that in each case the rate of oxidation is proportional to the amount of metal added over a fairly wide range. The temperature coefficient of the reaction was calculated from the velocities at 10° and at 20° in the presence of 0·0013 mg. Cu and of 0·002 mg. Fe and was found to be 2·0 (± 0·1) in the first case and 2·2 in the second.

The catalytic effects of iron and copper are sharply differentiated from each other by the different effects of variations in the concentrations of cysteine and H_2O_2. The rate of oxidation in the presence of copper is proportional to the peroxide concentration and independent of the amount of cysteine, whereas in the presence of iron it depends on cysteine and is independent of peroxide. (Curves illustrating this phenomenon in the case of iron are given later when the effect of phosphate is considered.) This effect is somewhat different from that studied by Elliott [1930] in the aerobic oxidation of cysteine; in this case the rate of oxidation depended on the cysteine concentration for both iron and copper catalysis. Elliott also found that, with 8 mg. of cysteine hydrochloride and at p_H 7·3, 0·0042 mg. Cu caused maximum rate of oxidation. It is apparent from Fig. 1 that there is no such limit in the case of oxidations at a lower p_H.

Effect of p_H. The effect of an increase in p_H on the catalytic oxidation is complicated by the fact that the rate of oxidation in the absence of added metal is also greatly increased and, at neutrality, is much too rapid to be followed conveniently by this technique. I have worked mainly at p_H 4·6.

1 cc. of 1 % cysteine hydrochloride was conveniently buffered at this p_H by 0·3 cc. of $M/3$ metal-free ammonium acetate, which was prepared by neutralising redistilled acetic acid with distilled ammonia. It is clear from Fig. 2 that this change in p_H has affected the rate of oxidation in the absence of added metal or in the presence of iron to a greater extent than that in the presence of copper. This suggests that the unavoidable oxidation is due to the presence of traces of iron. It is not of course permissible to compare these rates of change of rotation directly, for the specific rotations of cystine and cysteine vary with p_H. Cysteine in acid solution has a small positive rotation whereas

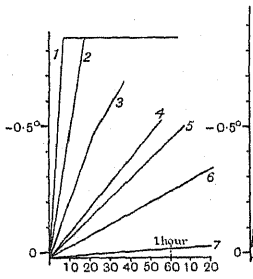


Fig. 1.

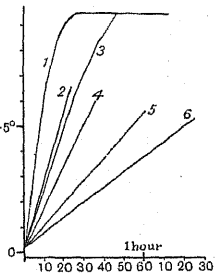


Fig. 2.

Fig. 1. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2·1.

1.	1 cc. 1 % cysteine-HCl;	0·02 mg. Cu;	0·5 cc. 0·44 N H_2O_2.
2.	,,	0·05 mg. Fe;	,,
3.	,,	0·003 mg. Cu;	,,
4.	,,	0·001 mg. Fe;	,,
5.	,,	0·001 mg. Cu;	,,
6.	,,	0·0005 mg. Fe;	,,
7.	,,	No metal	,,

Fig. 2. Volume made up to 5 cc. in each case. Temperature 20°. p_H 4·6.

1. 1 cc. 1 % cysteine-HCl; 0·001 mg. Fe; 0·3 cc. $M/3$ ammon. acetate; 0·5 cc. 0·44 N H_2O_2.
2. ,, 0·0003 mg. Fe; ,, ,,
3. ,, 0·001 mg. Cu; ,, ,,
4. ,, No metal; ,, 2·0 cc. 0·44 N H_2O_2.
5. 2 cc. cysteine-HCl; 0·6 cc. ammon. acetate; 1 cc. H_2O_2 incinerated *etc.* then 1 cc. cysteine-HCl; 0·3 cc. ammon. acetate and 0·5 cc. H_2O_2.
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ ammon. acetate; 0·5 cc. 0·44 N H_2O_2.

Bergmann and Michalis [1930] found that cysteine itself was slightly laevorotatory in aqueous solution. I find that this shift occurs at about p_H 4, and it is shown by a 0·04° change in the initial rotations in Figs. 1 and 2. Cystine undergoes a change in rotation at about the same p_H. Andrews [1925] found that the specific rotation of cystine, dissolved in hydrochloric acid, increased with the dilution. Lutz and Jirgensens [1930] showed that, with the same concentration of cystine, the rotation increased as the concentration of the acid or alkali used to dissolve it diminished. On account of its insolubility the rotation of cystine was not measured by these workers at neutrality. Supersaturated solutions of cystine are however relatively stable and, as Fig. 2 shows, the rotation of a very highly supersaturated solution of cystine does not alter for at least 40 minutes. By this method the specific rotation

of cystine for mercury green light and in 0·2 % solution has been determined at varying p_H.

p_H	$[\alpha]^{20}_{5461}$	p_H	$[\alpha]^{20}_{5461}$
2·1	$-276° \pm 2$	5·5	−312
2·5	−279	7·7	−297
3·1	−286	8·3	−227
4·6	−312	9·0	−156

In a later section when the effects of various inhibitors are considered the reason for the fairly high rate of oxidation in the absence of added metal will be discussed more fully. It has already been shown that the effect of p_H on the rate suggests that it is a case of iron catalysis. Curve 4, Fig. 2, shows however that at least the greater part of it is not due to iron, since a fourfold increase in H_2O_2 concentration increases the rate threefold, whereas control experiments show that at p_H 4·6, as at p_H 2·1, the rate of oxidation in the presence of iron is independent of the concentration of H_2O_2.

A similar conclusion was reached by testing the reagents separately for catalytic power after incineration. The following method was found to be the most reliable. A silica test-tube (10 × 1·5 cm.) was heated with a very small oxy-coal gas flame at a point a little to one side of the bottom and then drawn out with a piece of silica rod to form a horn 2 mm. long and 1·5 mm. broad at the base. The tube was clamped in a sloping position with the horn downwards and heated with a micro-burner giving a flame about 1·5 mm. broad. If care were taken that only the tip of the horn was touched by the flame the incineration proceeded smoothly without any bumping. Sulphuric acid and ammonia were freed from metal by distillation although ordinary pure ammonia (sp. gr. 0·880) is generally sufficiently free from metals already. The results of these experiments are somewhat inconclusive on account of the danger of contamination during the fairly lengthy incineration which is necessary with such a low metal concentration. 0·1 cc. of concentrated sulphuric acid was used and one drop of strong ammonia solution (sp. gr. 0·93) was added before the mixture was heated, this reduced the time necessary for complete incineration to about 15 minutes. The mixture was cooled, and 2 cc. of water were added followed by slight excess of ammonia. The excess ammonia was removed by cautiously boiling till ammonium sulphate crystallised. 1 cc. of cysteine hydrochloride solution was now added and, after a minute or two, 0·3 cc. of $M/3$ ammonium acetate, 0·5 cc. of 0·44 N H_2O_2 and the volume made up to 5 cc. Curve 5 on Fig. 2 is the result of incinerating 2 cc. of cysteine hydrochloride, 1 cc. of H_2O_2 and 0·6 cc. of ammonium acetate in this way. Similar experiments on the reagents separately showed that the greater part of this metal came from the cysteine hydrochloride. Ammonium sulphate at this concentration is without effect on the rate of oxidation either in the presence or the absence of added metal, though higher concentrations, as for example when 0·6 cc. of sulphuric acid is used for the incineration, inhibit the oxidation in both cases.

At p_H 1·6 the rate of oxidation in the presence of copper is only slightly less than at p_H 2·1, whereas the oxidation due to iron is greatly diminished. At this p_H cystine is fairly soluble, the effect of added cystine was therefore determined. Elliott [1930] has shown that, at p_H 7·3, cystine catalyses the copper but not the iron catalysis of cysteine oxidation. It is apparent from Fig. 3 that it catalyses neither type of oxidation appreciably at p_H 1·6. Metal-free cystine hydrochloride was made by precipitation with hydrogen chloride as described by Gerwe [1931]. A 1 % solution of this in $N/9$ HCl was used. In plotting the rotations measured in the presence of cystine, 0·86°, the rotation due to the cystine, has been subtracted in each case. Curves

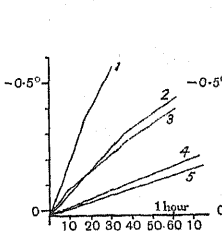


Fig. 3.

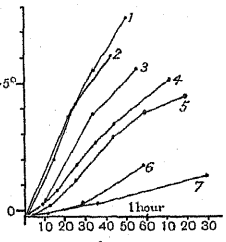


Fig. 4.

Fig. 3. Volume made up to 5 cc. in each case. Temperature 22°. p_H 1·6.

1. 1 cc. 1 % cysteine-HCl; 1 cc. $N/9$ HCl; 0·01 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
2. ,, 1 cc. $N/9$ HCl; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
3. ,, 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
4. ,, 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0·001 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
5. ,, 1 cc. $N/9$ HCl; 0·001 mg. Fe; 0·5 cc. 0·44 N H_2O_2.

Fig. 4. Volume made up to 5 cc. in each case. Temperature 19°. p_H 2·1.

1. 2 cc. 1 % cysteine-HCl; 0·002 mg. Fe 0·88 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·002 mg. Fe; no phosphate; ,,
3. ,, ,, 0·44 mg. KH_2PO_4; ,,
4. ,, ,, 0·88 mg. KH_2PO_4; ,,
5. ,, ,, ,, 1·0 cc. 0·44 N H_2O_2.
6. ,, 0·001 mg. Fe; 0·44 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
7. ,, 0·002 mg. Fe; 2·2 mg. KH_2PO_4; ,,

showing the effect of cystine on iron catalysis in the presence of phosphate are given later.

Effect of phosphate. Copper catalysis is unaffected by even $M/100$ phosphate both at p_H 2·1 and 4·6. Iron catalysis on the other hand is strongly inhibited at both reactions. This is shown in Fig. 4. The stock phosphate solution was $M/62$ KH_2PO_4, and even 0·2 cc. *i.e.* $M/1550$, was strongly inhibiting. As curves 1, 4 and 5 show, the rate of oxidation in the presence of iron, even when it is partially inhibited by phosphate, depends on the cysteine concentration and is independent of the concentration of H_2O_2. The slight inhibition shown in curve 5 has been obtained several times and becomes large if a large amount of H_2O_2 is added. In the presence of 0·00064 M phosphate the rate of oxidation, during the first hour at any rate, is no longer proportional to the iron present. In the presence of phosphate, therefore, cysteine behaves like thioglycollic acid [Elliott, 1930] so far as iron catalysis is con-

cerned. The situation is, however, complicated by the autocatalytic shape of the curves. Dixon and Tunnicliffe [1922] observed this autocatalysis in the methylene blue oxidation of .SH groups at p_H 7·6 but not at p_H 6. Fig. 5 shows that it is not due to catalysis by the cystine formed for, in the presence of iron and at p_H 1·6, the oxidation is autocatalytic when phosphate is present but is not catalysed by cystine. The autocatalysis does not seem to be due to a difference between ferric and ferrous phosphate for the curves are the same whether the iron and cysteine are first mixed together and allowed to stand for 10 minutes before the addition of the phosphate and peroxide, or the iron and peroxide mixed first and then the other reagents added. It is possible, though from a quantitative point of view unlikely, that the increase in the

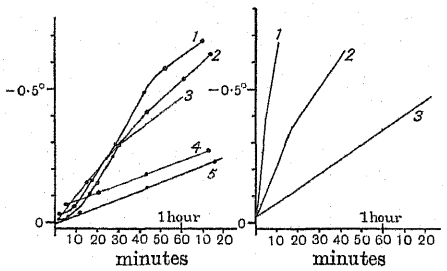


Fig. 5.

Fig. 6.

Fig. 5. Volume made up to 5 cc. in each case. Temperature 22°.

1. 1 cc. 1 % cysteine-HCl; 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0·01 mg. Fe; 0·66 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 1 cc. $N/9$ HCl; 0·01 mg. Fe; 0·66 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2. The reagents being added in this order.
5. 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·5 cc. 0·44 N H_2O_2; 1 cc. 1 % cysteine-HCl. The reagents being added in this order.

Fig. 6. Volume made up to 5 cc. in each case. Temperature 22°. p_H 4·6.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ ammon. acetate; 0·0005 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
2. „ ; „ ; 1·6 mg. KH_2PO_4; „ „
3. „ ; „ ; 2·2 mg. KH_2PO_4; no metal; „ „

rate of oxidation as the oxidation proceeds is due to the disappearance of H_2O_2 since the latter exerts a slight inhibitory effect. If this were the explanation, the initial rate of oxidation of a mixture, made up as in curve 4, Fig. 4, but with only 0·4 cc. of H_2O_2, should be the same as the rate after 20 minutes with 0·5 cc. Actually the two curves are identical.

At p_H 4·6 the inhibitory effect of phosphate is, as might be expected from the work of others, less marked. A pair of curves illustrating this is given in Fig. 6 and also a control curve in the presence of 0·0032 M phosphate. A comparison of this curve with the similar one in Fig. 2 shows that the inhibition is hardly outside the experimental error; this suggests, in agreement with the incineration results, that only a small part of the unavoidable oxidation can be due to iron.

Effect of pyrophosphate. Copper catalysis is unaffected by pyrophosphate unless the latter is present in very high concentration. Catalysis by iron on the other hand is inhibited quantitatively by pyrophosphate even when there is present only slightly more than one molecule of pyrophosphate for each molecule of iron. When there is a large amount of iron present (0·02 mg.) it can be inhibited by less than one molecule of pyrophosphate to one of iron, but a precipitate forms after a few minutes and makes the fluid in the tube opaque. No precipitate is formed in the presence of excess pyrophosphate. Fig. 7 shows the effect of pyrophosphate on iron catalysis at p_H 4·6, and it is clear

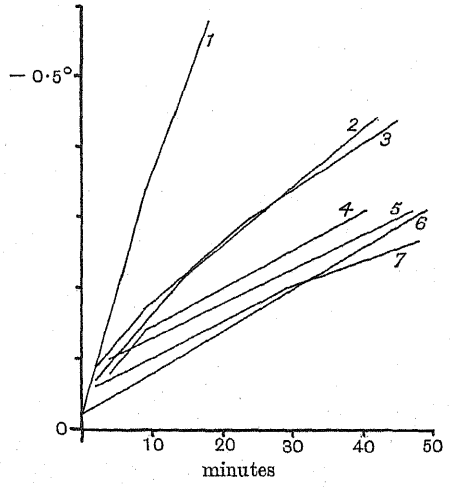


Fig. 7. Volume made up to 5 cc. in each case. Temperature 20°. p_H 4·6.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2; 0·0032 mg. $Na_4P_2O_7.10\,H_2O$.
2. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·008 mg. pyrophosphate; 0·5 cc. H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·062 mg. Fe; 0·0002 mg. Cu; 0·8 mg. pyrophosphate; 0·5 cc. H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·011 mg. pyrophosphate; 0·5 cc. H_2O_2.
5. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·016 mg. pyrophosphate; 0·5 cc. H_2O_2.
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal or pyrophosphate; 0·5 cc. H_2O_2.
7. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·062 mg. Fe; 0·8 mg. pyrophosphate; 0·5 cc. H_2O_2.

that copper may be detected by this technique in the presence of 300 times its weight of iron. The curves for iron in the presence of a substantial excess of pyrophosphate show that the latter has only a very slight effect on the "blank." Experiments with pyrophosphate but without added iron show that not more than 15 % of the unavoidable oxidation can be due to iron. The curves in Fig. 7 show that there is always some rapid oxidation at the beginning of each experiment. Two curves are given in Fig. 5 which indicate that this is due to a difference between ferric and ferrous pyrophosphate since it is not obtained when the cysteine is added last. Fig. 5 also shows that, in

the presence of 620 times its weight of iron, copper is somewhat inhibited by pyrophosphate.

Effect of HCN. Metal-free HCN was prepared by distilling a mixture of sodium cyanide and sulphuric acid and collecting the distillate in water. The results are summarised in Fig. 8. By comparison with Fig. 1 it is apparent that cyanide activates iron at p_H 2·1 while it has a hardly appreciable inhibitory action on copper. At p_H 4·6 on the other hand it inhibits small amounts of copper completely and has a slight inhibitory effect on iron. The difference between curve 2 on Fig. 2 and curve 1 on Fig. 8 is however not

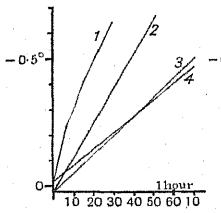


Fig. 8.

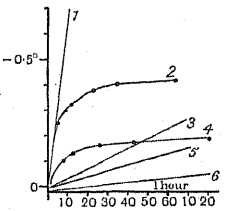


Fig. 9.

Fig. 8. Volume made up to 5 cc. in each case. Temperature 22°.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·0003 mg. Fe; 0·27 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·001 mg. Fe; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 0·001 mg. Cu; 1·0 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 *M*/3 ammon. acetate; 0·003 mg. Cu; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal; no HCN; 0·5 cc. 0·44 *N* H_2O_2.

Fig. 9. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2·1.

1.	1 cc. 1 % cysteine-HCl;	0·002 mg. Fe;	0·4 mg. ferrocyanide;	0·5 cc. 0·44 *N* H_2O_2.	
2.	,,	,,	1·0 mg.	,,	,,
3.	,,	no metal;	1·0 mg.	,,	,,
4.	,,	0·001 mg. Cu;	1·0 mg.	,,	,,
5.	,,	,,	0·2 mg. ferricyanide;		,,
6.	,,	,,	1·0 mg.	,,	,,

marked. A curve is also given in Fig. 8 showing that HCN has no effect on the rate of oxidation in the absence of added metal.

Effect of ferro- and ferri-cyanide. No attempt has been made to analyse fully the somewhat intricate systems to which these reagents give rise. They are slightly catalytically active themselves in the absence of added metal and, since the rate of oxidation seems to increase as the cyanide becomes more dilute, this is probably due to the decomposition of the cyanide. Fig. 9 shows that ferrocyanide activates iron at first and that this effect later gives place to an almost complete inhibition. Ferricyanide gives no such activation. Ferricyanide in the same way seems to inactivate copper almost completely at p_H 2·1 while ferrocyanide has first to be oxidised to ferricyanide (*cf.* curves 4 and 6 in Fig. 9). At p_H 4·6 the catalytic effect of these compounds masks any inhibitory effect which they may exert.

A number of other substances has been tested, using concentrations of about 1 mg. in 5 cc., as inhibitors for copper or iron. Carbon monoxide, urea, urethane, glutamine, alanine and histidine have no effect on copper; glutamic acid and potassium thiocyanate do not affect iron. The various reagents[1] introduced by Eichholtz [1929] and Krah [1930] were without effect on either metal when used in the maximum concentration possible with this technique. Owing to the colour of the reagents and to their insolubility in acid solution the concentration was generally 0·1 mg./cc. Sulphuretted hydrogen was converted immediately to free sulphur by the H_2O_2 present.

It was at first thought that low results which were often got with iron after incineration might be due to inhibition by pyrosulphate. The addition of pyrosulphate, however, inhibits neither iron nor copper at p_H 2·1 or 4·6. For some reason, therefore, the method of incineration used is not satisfactory if one wishes to estimate iron although it works perfectly in the case of copper.

Catalysis by copper derived from haemocyanin.

It is well known, from the work of Roche [1930] and others, that haemocyanin is not stable in acid solution. Haemocyanins from three species, *Helix pomatia*, *Limulus polyphemus* and *Maia squinado*, have been examined and in each case the copper appears to be completely liberated in catalytically active form at p_H 2·1. This, in the case of *Helix* is shown in Fig. 10, where the rates of oxidation in the presence of serum and of the same amount of serum after incineration are compared. This comparison has been made repeatedly and there has never been any difference between serum or dialysed serum and incinerated serum so far as catalytic activity is concerned. In only one sample of serum was there any detectable difference between the rates of oxidation in the presence and in the absence of pyrophosphate, the iron content of the sera of these three animals is therefore less than 1/20 of the copper content.

At p_H 4·6 neither *Helix* nor *Limulus* haemocyanin has any catalytic activity at all. In curve 6, Fig. 10, the crosses give the rate of oxidation in the absence of haemocyanin and the line gives two determinations of the rate in the presence of some snail haemocyanin. Curve 2 shows the effect of adding the same amount of haemocyanin to the cysteine hydrochloride before the ammonium acetate instead of after it. It makes no difference if the acetate is added 10 minutes after the haemocyanin instead of 20 seconds after, *i.e.* the decomposition at p_H 2·1 is complete in less than 20 seconds.

Maia haemocyanin is less stable than the other two and has a slight catalytic activity at p_H 4·6. Curves 3, 4 and 5 in Fig. 10 illustrate the decomposition which it undergoes in 3 minutes at p_H 3·9 and 3·65.

Since it is perfectly stable at p_H 3·3 this was the p_H used for the oxidation with *Helix* haemocyanin, the decomposition resulting from 3 minutes at

[1] 8-Hydroxyquinolinesulphonic acid, 1-amino-8-naphthol-4-sulphonic acid, 1-amino-2-naphthol-6-sulphonic acid, 5-nitro-4-chloro-2-aminophenol.

p_H 2·4, 2·7 and 2·9 is shown in Fig. 11. In these and all other cases the amount of ammonium acetate necessary to give any required p_H was found from a titration curve. $M/3$ ammonium acetate was run into 0·2 % cysteine hydrochloride solution and the p_H measured with a hydrogen electrode. Curves 1 and 2 on Fig. 11 show that, as might be expected, the rate of oxida-

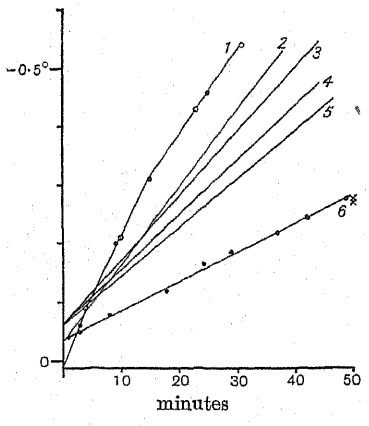


Fig. 10.

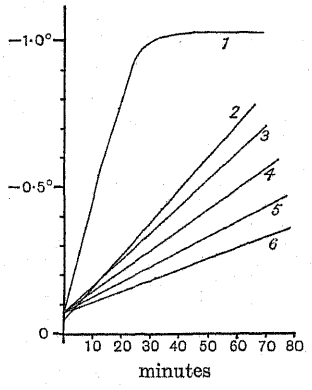


Fig. 11.

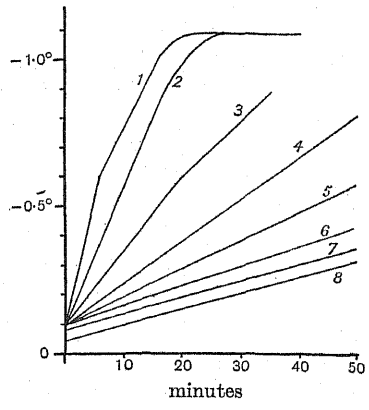


Fig. 12.

Fig. 10. Volume made up to 5 cc. in each case. Temperature 21°.

1. —.—.— 0·047 cc. of *Helix pomatia* serum; 1 cc. 1 % cysteine-HCl; 0·5 cc. 0·44 *N* H_2O_2.
   —o—o— 0·047 cc. of *Helix pomatia* serum incinerated; 1 cc. 1 % cysteine-HCl; 0·5 cc. 0·4 *N* H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·01 cc. *Helix* serum; 0·3 cc. $M/3$ ammon. acetate; 0·5 cc. 0·44 *N* H_2O_2.
3. 0·03 cc. of *Maia* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate and the H_2O_2 were then added — p_H 3·65
4. — p_H 3·9
5. — p_H 4·6
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ acetate; 0·01 cc. *Helix* serum; 0·5 cc. 0·44 *N* H_2O_2.

× × Extent of oxidation of controls after 50 minutes.

Fig. 11. Volume made up to 5 cc. in each case. Temperature 20°.

1. 1 cc. 1 % cysteine-HCl; 0·067 cc. *Helix* serum; 0·5 cc. 0·44 *N* H_2O_2.
2. " 0·017 cc. *Helix* serum; "
3. 0·017 cc. *Helix* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate, and the 0·5 cc. of 0·44 *N* H_2O_2 were then added. — p_H 2·4
4. — p_H 2·7
5. — p_H 2·9
6. — p_H 3·3
6. Control at p_H 3·3 with no haemocyanin.

Fig. 12. Temperature 20°. In each case the volume is 5 cc. and the final p_H is 4·6. An amount of *Limulus polyphemus* serum containing 0·0032 mg. of copper was added to each after the addition of the amount of ammonium acetate solution necessary to bring 1 cc. of 1 % cysteine-HCl to the required p_H. After a suitable time the rest of the ammonium acetate was added and then 0·5 cc. of 0·44 *N* H_2O_2.

1. 3 minutes at p_H 2·1.
2. 3 minutes at p_H 2·9.
3. 10 minutes at p_H 3·3.
4. 3 minutes at p_H 3·3.
5. 20 seconds at p_H 3·3.
6. 3 minutes at p_H 3·75.
7. 3 minutes at p_H 4·6.
8. Control at p_H 4·6.

tion at p_H 2·1 is proportional to the amount of haemocyanin. The effect of variations in the concentration of haemocyanin on the extent of its decomposition when the decomposition is only partial has not been measured.

In Fig. 12 curves are given illustrating the effect of p_H on the stability of *Limulus* haemocyanin. In 3 minutes a barely perceptible decomposition takes

place at p_H 3·75, but at lower p_H the decomposition is rapid. Three curves at p_H 3·3 show the effect of different times of exposure to acid. In all these experiments the actual oxidation takes place at p_H 4·6. Figs. 11 and 12 both show that when the oxidation is allowed to proceed to completion in the presence of decomposed haemocyanin the final rotation observed is more than 0·1° higher than that of the cystine formed. This extra rotation is due to the degraded haemocyanin. Natural haemocyanin has a small negative rotation which is doubled or trebled on the addition of acid, the addition of a corresponding amount of alkali does not reduce the rotation again to its old level. For example:

Rotation of 2 dm. of a solution of dialysed *Helix* haemocyanin containing 0·005 mg. of copper per cc.	$= -0·08°$
Rotation after the addition of 0·2 cc. of N H_2SO_4 to 10 cc. of haemocyanin. p_H now 1·8	$= -0·18°$
Rotation after adding 1 cc. of $N/5$ NaOH to this	$= -0·16°$

This last value was the mean of several readings made by two observers, since on neutralising a solution of haemocyanin which has been acid a very considerable turbidity develops. This was observed in all the experiments in which the stability of haemocyanin at varying p_H was measured, but, if the decomposition were not carried more than half-way, it is fairly slight.

Catalysis by haematin and haemoglobin.

The catalysis which is observed with these two substances is due, in part at any rate, to inorganic iron liberated under the conditions of the experiment. A few minutes' contact with H_2O_2 in the presence of cysteine hydrochloride is sufficient to discharge the colour of haemoglobin and, after this treatment, haematin will no longer give a haemochromogen with pyridine; both, on the other hand, now give an iron test with potassium thiocyanate. This decomposition takes place at both p_H 2·1 and 4·6.

The oxidation of glutathione.

In the presence of copper the oxidation of glutathione is very similar to that of cysteine. The rate of oxidation depends on the concentrations of copper and of hydrogen peroxide but it is independent of the concentration of glutathione. Thus curves 3 and 4 in Fig. 13 have the same gradient although the initial rotations are different since one represents only half as great a concentration of glutathione as the other. This rate of oxidation is doubled in curve 2, which has twice the H_2O_2 concentration, and in curve 1, with 0·006 instead of 0·002 mg. of copper, it is trebled. Meldrum and Dixon [1930] found that, for the aerobic oxidation of glutathione, small quantities of a third factor were necessary. Mason [1931] suggested that this was cysteinylglycine. This factor is of only secondary importance in the copper-catalysed oxidation by hydrogen peroxide. The sample of glutathione used in this work was very

pure, having been recrystallised from aqueous solution at about the freezing point. It gave theoretical analytical figures. A few cc. of the 2 % solution used were heated at 100° in a steam-bath for 35 minutes and, after cooling, used for oxidation experiments. The rate of oxidation in the presence of 0·006 mg. of copper or in the absence of added metal at p_H 4·6 was unaltered by this treatment although a considerable amount of decomposition is known to occur.

When the oxidation of glutathione is catalysed by iron it differs markedly from the oxidation of cysteine. The rate of oxidation is no longer proportional to the metal concentration but decreases suddenly when there is less than 1 part of iron for each 2000 of glutathione. Elliott [1930] found a very similar state of affairs in the iron-catalysed aerobic oxidation of thioglycollic acid.

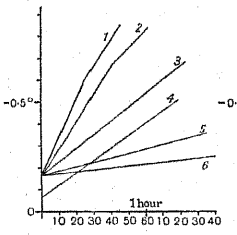


Fig. 13.

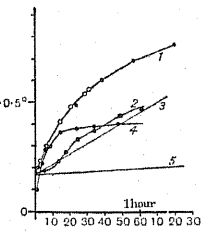


Fig. 14.

Fig. 13. Volume made up to 5 cc. in each case. Temperature 21°.

1. 1 cc. 2 % reduced glutathione; 0·006 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
2. 1 cc. 2 % reduced glutathione; 0·002 mg. Cu; 1·0 cc. 0·44 *N* H_2O_2.
3. 1 cc. 2 % reduced glutathione; 0·002 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
4. 0·5 cc. 2 % reduced glutathione; 0·002 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
5. 1 cc. 2 % reduced glutathione; no metal; 0·3 cc. *M*/3 ammon. acetate; 0·5 cc. 0·44 *N* H_2O_2.
6. 1 cc. 2 % reduced glutathione; no metal; 0·5 cc. 0·44 *N* H_2O_2.

Fig. 14. Volume made up to 5 cc. in each case. Temperature 21°.

1. —.—.—.— 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
   —o—o—o— 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 1·0 cc. 0·44 *N* H_2O_2.
2. 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2; 0·18 mg. KH_2PO_4.
3. 1 cc. 2 % reduced glutathione; 0·008 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
4. 0·5 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
5. 1 cc. 2 % reduced glutathione; 0·005 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.

As with cysteine the rate of oxidation is independent of the peroxide concentration but is, as would be expected, an inverse function of the glutathione concentration. These facts are illustrated in Fig. 14.

A preliminary heating of the glutathione to 100° seems to increase the rate of oxidation in the presence of iron. The effect is small however and, since any decomposition will result in a lowering of the concentration of glutathione and this has already been shown to cause an increased rate of oxidation, it may well be unconnected with the production of cysteinylglycine.

Inhibitors, so far as they have been studied, have a similar effect on both oxidations. Cyanide inhibits copper partially at p_H 2·8 while phosphate is without effect. Phosphate inhibits iron strongly and at certain concentrations (*M*/4000) the curve is slightly inflected (curve 2, Fig. 14).

Discussion.

The nature of the oxidation occurring at p_H 4·6 cannot be considered to be definitely settled but it seems very probable that all the oxidation at this p_H is not due to traces of metals. It behaves in a manner unlike iron catalysis so far as variations in H_2O_2 concentration are concerned and it is not inhibited by phosphate or pyrophosphate. It behaves on the other hand unlike copper catalysis towards variations in p_H and it is not inhibited by cyanide. Furthermore, if an experiment similar to that given in curve 5 of Fig. 2 is carried out, but with the addition of 0·1 mg. of pyrophosphate, the rate of oxidation is identical with that of the "blank" in the presence of pyrophosphate. This indicates that what metal there is, is iron. In a later paper I hope to describe the catalysis of this reaction by thiocarbamide. This oxidation will proceed rapidly in the absence of added metal and the effect of metal is, over a certain range at any rate, simply additive. The existence of this latter example of a "metal-free" oxidation by hydrogen peroxide lends colour to the idea that the present oxidation also can proceed in the absence of metal. At Prof. Haldane's suggestion the temperature coefficients of the copper- and iron-catalysed oxidations and of the oxidation without added metal were measured. In each case the coefficient was $2 \pm 0{\cdot}2$. No distinction between the three types of oxidation can therefore be drawn in this way.

At p_H 6 the oxidation of cysteine, without the addition of metal, is complete in a few minutes, the final rotation being 0·95° as in the case of the metal-catalysed oxidation. The final rotation when the oxidation occurs at p_H 4·6 cannot be determined since the cystine begins to crystallise after about 2 hours, *i.e.* before the reaction has gone to completion. Even in the presence of a very substantial excess of peroxide (1 cc. of 0·064 *M* cysteine hydrochloride or 1 cc. of 0·066 *M* glutathione mixed with 0·5 cc. of 0·44 *N* H_2O_2) the rotation of an acid solution of either cystine or oxidised glutathione will remain constant for over 12 hours, while the expected amount of hydrogen peroxide remains in the solution and can be estimated by titration. In acid solution therefore, in the presence of iron and copper and in the case of glutathione of the decomposition products formed by boiling for a short time, hydrogen peroxide can only react very slowly, if at all, to bring about further oxidation. On the alkaline side further decomposition readily takes place. In this connection the specific rotation of oxidised glutathione has been measured at several different reactions. Mercury green light was used and the solution, initially 1 %, was diluted somewhat at the higher p_H since in all 1·5 cc. of *N*/5 sodium hydroxide were added to 6 cc. of glutathione solution.

p_H 2·85	$[\alpha]^{21}_{5461}$ − 115° ± 1	p_H 7·0	$[\alpha]^{21}_{5461}$ − 133
5·0	− 125	8·6	− 150
6·0	− 126	9·6	− 176

Glutathione resembles cysteine in showing a slight increase in specific rotation as the p_H increases from about 3 to 5. Unlike cysteine, however, it shows a further increase as the p_H rises above 5.

It is obvious that this catalytic reaction offers a very convenient method for estimating small amounts of copper, if necessary in the presence of iron. The sensitivity is about the same as that of Warburg's method [1927] but the technique is easier and, since all the solutions are acid, vessels simply need to be rinsed out 3–4 times with glass-distilled water after use. With the polarimeter available in this laboratory readings are accurate to 0·01° (in general the observed points have only been put on those curves which are non-linear; if the points on the graphs had been given a diameter corresponding with this probable error they would all have touched the lines which have been drawn) quantities of copper of the order of 0·001 mg. may therefore be estimated with an error of about 5 %. Metals such as vanadium will of course interfere with this method of estimation.

Summary.

In the presence of copper the rate of oxidation of cysteine by hydrogen peroxide at p_H 2·1 is proportional to the concentrations of copper and peroxide but independent of that of cysteine.

In the presence of iron it is proportional to the concentrations of iron and cysteine and is slightly inhibited by an increase in H_2O_2.

At p_H 4·6 there is some metal-free oxidation and the rate of oxidation in the presence of iron is much increased while that in the presence of copper is less markedly affected.

Figures are given for the specific rotations of cysteine and glutathione between p_H 2·1 and 9·0.

Phosphate inhibits iron and gives an inflected oxidation curve when the inhibition is incomplete. Pyrophosphate inhibits iron stoichiometrically.

At p_H 2·1 cyanide activates iron and hardly affects copper, at p_H 4·6 both are inhibited. Ferricyanide inhibits copper and iron, but ferrocyanide activates iron and only inhibits copper when it has been oxidised.

Haemocyanin has no catalytic effect but at p_H 2·1 it is decomposed completely giving catalytically active copper. The stability of three different haemocyanins at varying p_H has been measured.

Haematin and haemoglobin are not stable under the conditions used.

The rate of oxidation of glutathione in the presence of copper is proportional to the concentrations of copper and H_2O_2 and independent of that of glutathione. In the presence of iron there is a sort of auto-inhibition and the rate of oxidation is an inverse function of the glutathione concentration; it is independent of the H_2O_2.

This reaction is suggested as a convenient method for estimating small amounts of copper.

I am deeply indebted to Mrs K. G. Terroux for the various samples of haemocyanin and to Sir F. G. Hopkins for his interest and advice.

REFERENCES.

Abderhalden and Wertheimer (1923). *Pflüger's Arch.* **199**, 336.
Andrews (1925). *J. Biol. Chem.* **65**, 147.
Bergmann and Michalis (1930). *Ber. deutsch. chem. Ges.* **63**, 987.
Dixon and Tunnicliffe (1922). *Proc. Roy. Soc. Lond.* B **94**, 266.
Eichholtz (1929). *Arch. exp. Path. Pharm.* **148**, 369.
Elliott (1930). *Biochem. J.* **24**, 310.
Gerwe (1931). *J. Biol. Chem.* **91**, 57.
Krah (1930). *Biochem. Z.* **219**, 432.
Lutz and Jirgensens (1930). *Ber. deutsch. chem. Ges.* **63**, 456.
Mason (1931). *J. Biol. Chem.* **90**, 25.
Meldrum and Dixon (1930). *Biochem. J.* **24**, 472.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1931). *Biochem. J.* **25**, 614.
Quastel, Stewart and Tunnicliffe (1923). *Biochem. J.* **17**, 586.
Roche (1930). *Arch. Physique Biol.* **7**, 207.
Warburg (1927). *Biochem. Z.* **187**, 255.

CLXXI. THE ABSORPTION OF WATER BY GELATIN.

VI. THE INFLUENCE OF THE THICKNESS AND ORIGINAL CONCENTRATION OF THE GEL.

By DOROTHY JORDAN-LLOYD.

From the Laboratories of the British Leather Manufacturers' Research Association.

(*Received August 13th, 1931.*)

Introduction.

In the previous papers in this series on the behaviour of dry leaf gelatin when immersed in watery media, the effects of variations in the composition of the external medium have been studied [Jordan-Lloyd, 1920, 1930; Jordan-Lloyd and Pleass, 1927, 1928; Pleass, 1929, 1930]. It is, however, obvious that if (as frequently postulated in the course of the work) gelatin gels are systems with a definite internal architecture, variations in the methods used in preparing the dry gelatin might also be expected to influence the behaviour of the system. It has indeed been recognised from the earliest days of investigations into the physical chemistry of gelatin that the characteristics of the particular sample of gelatin used must be taken into consideration. Hofmeister [1890] and Pauli a few years later [1897] both introduced a factor allowing for the thickness of the dry gelatin in equations expressing the rate of swelling of gelatin in water. The idea that both external and internal dimensions of the dried gelatin play a part in its subsequent behaviour in water has indeed been latent in much of the literature, the ratio of surface to mass, for instance, having been considered by other workers besides Hofmeister and Pauli. The problem of the influence of the internal structure of the gelatin has, however, only been investigated in comparatively recent times.

Procter [1911] showed that the swelling of dried gelatin gels in water was partly controlled by the percentage concentration at which the original gelatin jelly had been allowed to set. Using gelatin dried from 5, 10 and 20 % jellies, he showed that swelling in water was inversely proportional to the original concentration. Gortner and Hoffmann [1922] confirmed this observation; using gelatin gels dried from jellies of 2·5, 5, 10, 15 and 20 % concentration, they showed that both the rate and amount of swelling in water were inversely proportional to the concentration at setting. Their experimental figures also give an indication that in contact with water the 10 % gel has a stability of concentration greater than that of gels which are more concen-

trated or more dilute, for, while gels dried down from more concentrated jellies regain more water on soaking than they lost during evaporation, and gels dried down from less concentrated jellies do not regain as much water as they lost, the gel dried from a 10 % jelly returns approximately to its former state on soaking in water. The special stability of the 10 % gelatin jelly has been demonstrated in a very attractive manner by Kunitz [1928], who used jellies of different gelatin concentrations from 3 to 20 % and immersed them without previous drying in $M/1000$ acetate buffer solutions at p_H 4·7. All the jellies which contained more than 10 % of gelatin absorbed water and all those which contained less gave up water to the surrounding fluid. Northrop [1927] and Northrop and Kunitz [1927] had previously shown that the equilibrium weight of gelatin jellies in water could be expressed by an equation involving the concentration at setting as a variable, and Kunitz, in his later paper, showed that the equation applied equally whether that equilibrium were attained by either gaining or losing water.

The work reported in the present paper deals with the influence of both thickness and original concentration of the jelly on the swelling of gelatin gels in water and in salt solutions, and at acid and alkaline reactions. The experimental evidence shows that both the thickness of the gelatin pieces and the original concentration of the gels at setting influence the amount of water absorbed at equilibrium by gels under different external conditions. This is equally true whether the gels are placed in the experimental conditions in the undried state or whether they have been taken to dryness as a first stage. The experiments, however, also show that the process of drying the gels at temperatures below their melting points very greatly reduces the capacity to absorb water under all the experimental conditions studied (the influence of the drying being inversely proportional to the original concentration) and that, therefore, changes occur in the gelatin gels during drying, which are not fully reversed on restoring to an aqueous medium.

Method and materials.

Most of the experiments were carried out with Coignet's Gold Label Gelatin, purified by the method previously described [Jordan-Lloyd and Pleass, 1927], but as large quantities of material were required, the commercial gelatin was sometimes used without purification. The latter possesses the practical advantage over the purified gelatin of being sufficiently soluble to make a 50 % gel. The gelatin used is, in every case, indicated in the experimental records. The gelatin was dissolved by soaking in cold water and then warming to 50° and adjusting the volume to the required concentration. Gels were cast by pouring the warm sol into moulds of hard paraffin wax and, when required for use in the dry condition, were dried as rapidly as possible at room temperature under an electric fan.

The experiments were carried out by placing the weighed gelatin pieces in stoppered glass jars with the experimental solution and a small amount

of toluene to prevent bacterial growth. Water absorption was followed in every experiment by weighing at suitable intervals of time until equilibrium (or substantial equilibrium) had been attained. Time curves are not given in this paper, but equilibrium may be considered to be reached at 23° in 3 days, at 18° in 4 days, at 7° in 7 days, at 0° in 10 days. Gels of pure gelatin come to a true equilibrium at 0° and 7°, but continue to absorb water slowly for an indefinite period at higher temperatures, due possibly to the solubility of the gelatin at these temperatures or else to the development of "fatigue" [see Northrop, 1927]. Gels of commercial gelatin do not come into a true equilibrium at any temperature, though at low temperatures they come to a state in which further changes are slow. In most cases, the absorption of water from (1) distilled water, (2) 0·2 M sodium chloride solution, (3) N/1000 hydrochloric acid, (4) N/1000 sodium hydroxide was followed at various temperatures. In some cases, experiments on absorption of water from salt solutions were extended to include a range of concentration from 0·1 to 2·0 M and also to include sodium nitrate and sulphate as well as chloride.

Experiments on the thickness of the gelatin.

Experiments on the influence of the original thickness of the experimental pieces were carried out, both on undried and on dried gels. For comparative purposes, the thickness of the undried gels was not measured in the undried condition but was measured from the unused portion of the experimental castings after drying. Gels of 5, 10 and 15 % original concentration were used in the experiments on thickness and a range of from 0·06 to 0·65 mm. of dry thickness was covered. Thin sheets of gelatin cannot be cast from the 15 % gels since the surface forces prevent the warm sol from spreading over the wax moulds unless a minimum thickness can be attained. Experimental results at 0° both for dried and undried gels in water, salt, acid and alkaline solutions are shown in Fig. 1 (*a*) and (*b*). It can be seen that with water and sodium chloride solution the effect of the thickness is comparatively slight, although in all cases the thin pieces contain a higher percentage of water than the thick ones. With the acid and alkaline solutions, however, the thickness of the pieces has a very considerable influence on the amount of water absorbed. In Fig. 1 (*c*) these swelling curves for undried 5 % gels have been plotted against the logarithm of the thickness and it can be seen that the points lie on two straight lines. The same relation holds for the 10 and 15 % gels. Hence the relation of swelling to the thickness of the pieces can be expressed by the equation:

$$S = -k_1 \log d + k_2,$$

where S is the swollen weight at equilibrium, d is the dry thickness and k_1 and k_2 are constants. The values of k_1 and k_2 depend partly on the original concentration of the gel and partly on such factors as p_H and temperature. For any particular value of the initial concentration, it seems highly probable

that $k_2 = f(k_1)$, under conditions including both acid and alkaline swelling. There are not, however, sufficient experimental data to establish this definitely.

Further, experiments on the influence of thickness on water absorption of gels after drying bring out two points clearly: firstly, that under similar experimental conditions very much less water is absorbed than by undried

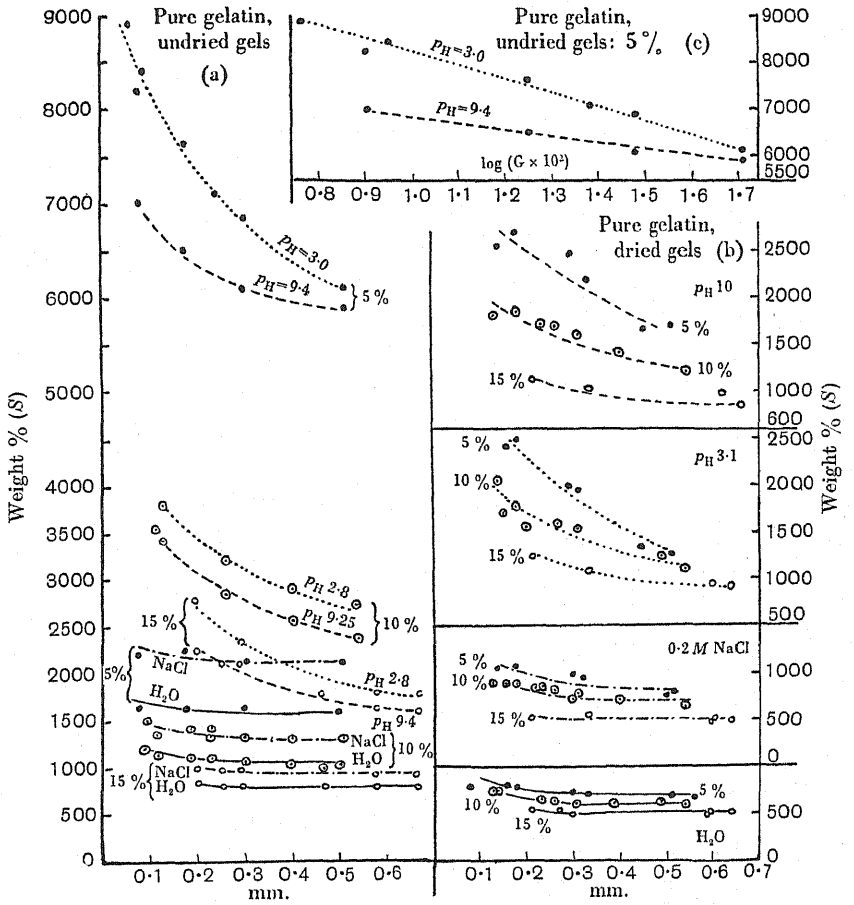


Fig. 1.

gels; secondly, that the influence of thickness of the gels has become less in the case of the acid and alkaline systems. The equation connecting swelling and thickness of the gel in water and salt solutions over the range of thickness covered by these experiments approximates to the form

$$S = -k_3 d + k_4.$$

The curve for swelling in acid and alkaline solutions also approximates to the linear form, though obviously the value for k_3 is considerably greater than in the case of water and salt solutions.

The effect of drying the gel is a little difficult to explain, but it is clear that the lower the original concentration of the gel, the greater the effect of the drying. It, therefore, seems possible that during drying there is a re-organisation of the molecules of gelatin and of water within the gel of such a kind that the dried gel corresponds in internal structure to a more concentrated system than the jelly from which it has been dried. A re-arrangement of this type would lead to a reduction in the amount of water that would be absorbed on re-soaking. Details of the relation between swelling and the concentration of gelatin in the jelly are dealt with in the next section.

The effect of the initial concentration of the gel.

Experiments were made both with the original freshly set jellies and with gels which had been brought to the dry condition. The water absorption of both types was studied under various conditions. Experiments with pure gelatin were made at 5, 10, 15 and 20 % original concentration; experiments with commercial gelatin were made at 5, 10, 20 and 50 % original concentration. Observations were made at 0°, 7°, 18° and 23°. The factor for thickness was controlled, 0·25–0·30 mm. being usually chosen.

The freshly set jellies in water at 0° reach a steady equilibrium in the course of a few days, which remains steady up to 3 weeks, beyond which their behaviour was not followed. The 5 % gel lost weight; the 10 % changed very little, generally, however, showing a slight gain; the 15 and 20 % gels both gained in weight (see Figs. 1 (*a*) and 2 (*a*)). At all other temperatures all the gels gained in weight. In 0·2 *M* sodium chloride solution at 0° the gels also reach a steady equilibrium. Under these conditions the 5 % gel shows very little change of weight; the other gels all gain. At higher temperatures all gain. In dilute hydrochloric acid and sodium hydroxide solutions all gels absorb water freely at 0° and 7° and pass into solution at 18° and 23° after a few days.

The weight gained under various conditions, plotted against the original concentration, is shown in Fig. 2 (*a*). Under all circumstances, the total amount of water in the gel when it is in substantial equilibrium with its surroundings is an inverse logarithmic function of the original concentration. In Fig. 2 (*b*) the same experimental data shown in Fig. 2 (*a*) have been plotted against $1/\log G$ as abscissa, where G is the original concentration of the gelatin in the jelly. It can be seen from both figures that all the curves of swelling against concentration form a single family of curves in spite of variations of temperature and of the nature and concentration of the electrolyte in the system. The influence of initial concentration on swelling can, therefore, be expressed by the general equation:

$$S = k_5\left(\frac{1}{\log G}\right) + k_6,$$

where S is the swelling, G the original concentration and $k_6 = f(k_5)$.

Swelling, therefore, is uniformly modified under all conditions by the

properties of the gel. These properties remain fundamentally the same under different physical and chemical conditions and are, therefore, to be attributed to the mechanical properties of the gel. Without any great straining of probabilities, the influence of concentration on swelling may be attributed to the mechanical resistance of the tangled micellae in the gel to being moved, either further apart or nearer together. If the effect of concentration on swelling under any particular conditions be considered, it follows from relations of the curves to each other that the resistance to swelling diminishes not only with a fall in the initial concentration, but also under all conditions which

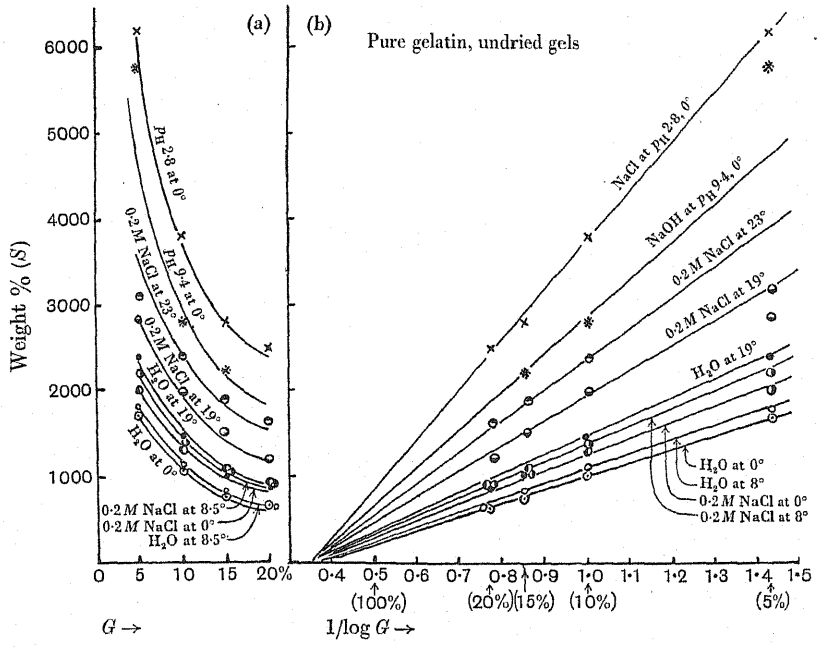


Fig. 2.

lead to an increase in the amount of water present in the gel, or in other words, the ease with which the micellae are pushed apart increases with the distance between them.

The influence of the original concentration of the gelatin in dried gels is shown in Fig. 3 (*a*) and (*b*). Curves for swelling plotted against $1/\log G$ are given both for pure gelatin (Fig. 3 (*a*)) and for commercial gelatin (Fig. 3 (*b*)). As in the experiments on thickness, the most striking effect of drying the gels before putting them under the different experimental conditions is that under all circumstances the amount of water absorbed into the system is very considerably reduced, especially in the weaker gels. As long as the original concentration of the gelatin is not greater than 20 %, the general effect of concentration on swelling can still be expressed by the general equation:

$$S = k_5\left(\frac{1}{\log G}\right) + f(k_5),$$

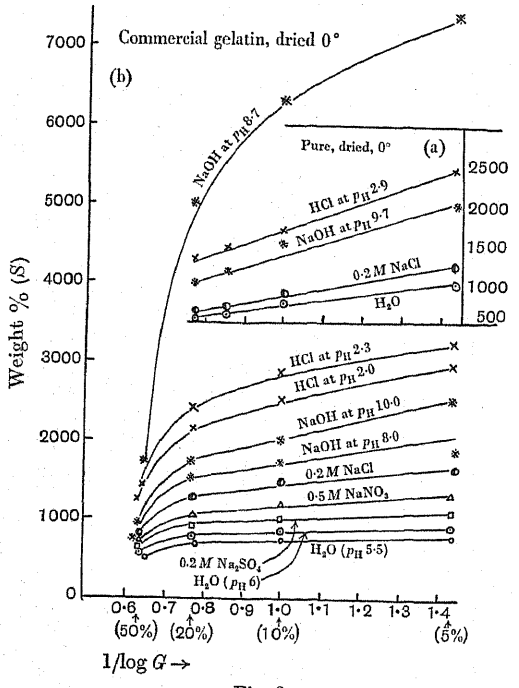


Fig. 3.

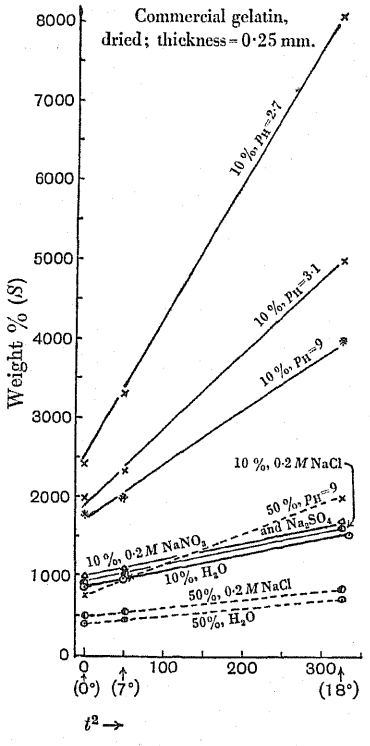


Fig. 4

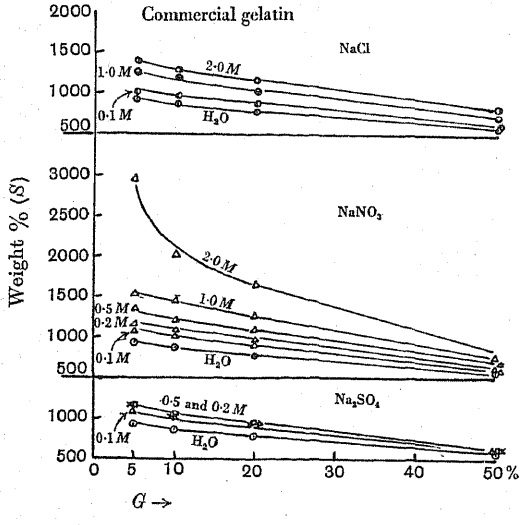


Fig. 5.

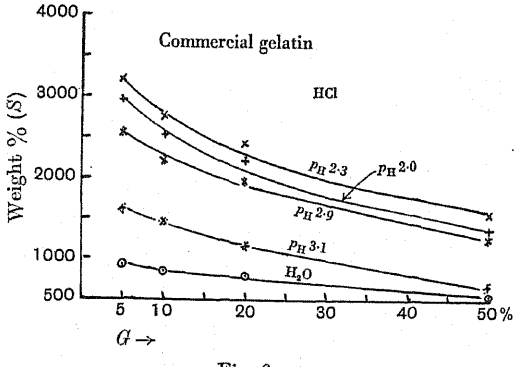


Fig. 6.

but the values of the constants k_5 and $f(k_5)$ have become different. The points obtained for the 50 % (commercial) gels do not, however, lie on the curve calculated from the general equation. It seems highly likely that both in undried jellies and dried gels this equation does not hold if the original concentration of the gelatin rises above about 20 %.

The curves for the swelling of the commercial gelatin cover a wide range of external conditions. The swelling of the gelatin has been followed not only for sodium chloride, but also for sodium nitrate and sodium sulphate. Further experimental data for these three salts over a range of concentrations up to $2M$ are given in Fig. 5. It can be seen that the influence of concentration on the water absorption remains similar with all three salts over the range of concentration studied. The values of the constants in the general equation will, however, be affected by the nature and concentration of the salt. Considering each salt individually, it is again found that the curves of swelling for different concentrations of salt form a family of curves which, if extended, come to a point at a value of $S = 100$, $G = 100$; *i.e.* under conditions where the gel contains no water, the presence of salt would fail to cause swelling. The same relation holds for the series of curves of swelling in hydrochloric acid at different values of p_H (Fig. 6).

The effect of temperature.

The effect of temperature on water absorption by gels of different thicknesses and original concentration have been studied both for the undried jelly and the dried gelatin. Experimental data are given in Fig. 7. For all systems studied, *i.e.* for water, salt, acid and alkaline solutions, the curves of weight plotted against temperature are true parabolas over a temperature range of 0°–18°, *i.e.* $S \propto t^2$, where t is the temperature in degrees centigrade. The curves for the pure gelatin gels (undried and dried) are not shown with S plotted against t^2, but Fig. 4 shows the data for commercial gelatin under a number of experimental conditions plotted on this system. The resulting curves are linear, hence under all conditions

$$S = k_7 t^2 + k_8,$$

where t is the temperature (centigrade) within the limits 0° to 18°. Where the experimental curves depart from the general parabolic form as the temperature rises, the gelatin has begun to dissolve in the surrounding medium. The relative positions of the curves (Fig. 7) suggest that the constant k_8 is influenced by both the thickness and the original concentration of the gel, but these two factors do not influence the value of the partial differential dS/dt, except possibly when the gels were set either at a low percentage of gelatin or in a very thin sheet. (In this connection, it will be noticed, on comparing the data given here with those published in the previous papers of the series, that a parabolic curve was not previously obtained for variations of swelling with temperature in water (p_H 5·0) and very dilute alkalis (p_H 7·0).

The sheet gelatin used in the previous work was obtained by dialysing commercial gelatin and was very thin, the average thickness of the pieces being 0·06 mm. compared with the 0·20–0·30 mm. used in the present work. The relatively greater surface exposed to solvent action is probably sufficient to account for the difference in experimental data.)

The value of the constant k_7 is evidently influenced by the nature of the electrolytes in the system, being very much greater in acid or alkaline than in water or salt solutions (Figs. 4 and 7). The value of the constant k_7 does not appear to be influenced by drying the gel, while the value of k_8 is considerably affected. The suggestion already put forward in this paper that a re-organisation occurs in the gel during drying in such a way as to bring

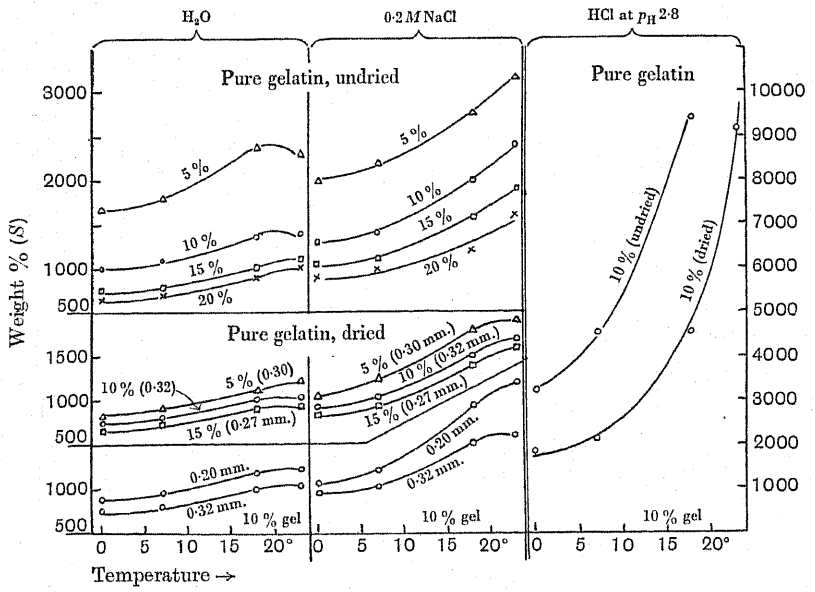


Fig. 7.

about an approximation in structure to one which set at a higher concentration of gelatin, receives support from the influence of drying on the value of k_8, which, it has already been shown, is influenced by the original concentration.

The influence of temperature on swelling is, however, so similar in nature under all external conditions that it seems clear that the predominating effect of the temperature is on some factor common to all the systems; this factor might be the gelatin itself or it might be the water. It has already been shown by Smith [1919] that gelatin in solution in water undergoes a reversible mutarotation with change of temperature. Below 15°, the "gel" form, gelatin B with $[\alpha]_D = 141°$, is the only stable form; at temperatures above 35°, the "sol" form, gelatin A with $[\alpha]_D = -313°$, is the only stable form. This transformation of the gelatin with change of temperature might be considered

responsible to some extent for lowering of the cohesive forces of the gelatin which occurs with rise of temperature, but from Smith's experimental figures only 3 % of gelatin B will be present at 18°, while from the results recorded in this paper, as soon as the concentration of gelatin B rises above this figure, solution of the gelatin sets in and equation $S = k_7 t^2 + k_8$ no longer holds. The mutarotation of gelatin with temperature, therefore, is not a factor in this equation. The influence of the temperature on the activity of the water is, therefore, probably to be regarded as the direct cause of the influence of temperature on swelling. The fact that $S \propto f(t^2)$ on the centigrade scale, where the zero is based on the property of water, is noteworthy in this connection.

In acid and alkaline solutions temperature has such a profound influence over the whole range that there seems little doubt that it influences not only the activity of the water but also the osmotic and other properties of the gelatin salts formed under these conditions.

The special stability of the 10 % gel.

It has already been mentioned in the introduction to this paper that under certain conditions 10 % gels are more stable than those of any other concentration. This observation, originally recorded in the literature by Kunitz for experiments made at 5°, is fully confirmed by the experiments recorded here. The stability of the gelatin jellies in a system free from electrolytes is not independent of either the temperature or the purity of the gelatin. It has been observed constantly throughout this work that commercial gelatin never comes into true equilibrium with its surroundings, but after nearly attaining equilibrium continues to absorb water and gain in weight for an indefinite period. The lower the temperature, the more nearly is a true equilibrium reached, and an approximate equilibrium may be taken as a measure of the change in weight due to imbibition. With pure gelatin, a true equilibrium is reached at low temperatures (0° and 7°) but not at higher ones (18° and 23°), a situation possibly to be attributed partly to the appearance of Smith's soluble B form of gelatin at the higher temperatures. The rate at which the end of the true swelling of the gelatin is reached varies with the temperature, but the following times were found by experiment to represent the end of the first rapid water absorption: 0° in 10 days, 7° in 7 days, 18° in 4 days, 23° in 3 days.

Table I shows the weights attained in equilibrium in water by gels which had been set over a range of concentration from 5 to 50 %. Figures are given both for undried jellies and gels after drying.

It will be seen here again that, at the low temperatures, all gels tend to move towards the final concentration of 10 % or 1000 parts by weight of swollen gelatin to every 100 parts of gelatin in the system. At the higher temperatures, where imbibition is undoubtedly followed by slow solution, all gels tend to draw water into the system.

Table I.

Concentration of jelly at setting ...	5 %	10 %	15 %	20 %	50 %
Original weight of jelly for 100 g. gelatin (g.)	2000	1000	670	500	200
	Weight of gel after soaking in water for 100 g. gelatin (g.)				
Pure gelatin jellies (*undried*) (dry thickness 0·24–0·28 mm.)					
10 days at 0°	1700	1050	760	550	—
7 ,, 7°	1800	1070	800	570	—
4 ,, 18°	2300	1350	830	810	—
3 ,, 23°	∞	∞	1070	1000	—
Pure gelatin gels (*dried before soaking*) (thickness 0·27–0·32 mm.)					
10 days at 0°	1100	750	700	—	—
7 ,, 7°	1150	800	770	—	—
4 ,, 18°	1100	970	950	—	—
3 ,, 23°	1200	1050	1000	—	—
Commercial gelatin gels (*dried before soaking*) (thickness 0·22–0·25 mm.)					
10 days at 0°	920	850	—	750	450
7 ,, 7°	1020	950	—	800	470
4 ,, 18°	1750	1500	—	1250	750
3 ,, 23°	2000	1700	—	1600	900

The special stability of the 10 % gel is shown most clearly in systems which have not been dried. After drying, the re-organisation which has occurred has partly masked the effect of the original concentration. The special stability of the 10 % gel is not apparent in systems where electrolytes are present. The presence of acid, alkali or salt alters the equilibrium between the gelatin and the water. There is some evidence, however, that the tendency for gels of originally higher or lower concentration is to move in their final equilibrium towards the final equilibrium of the 10 % gel.

Discussion.

The fact that gelatin jellies absorb water and swell in aqueous solutions until they have reached an equilibrium weight has always been recognised as evidence of cohesive forces due to the gelatin itself [Procter, 1914]. These cohesive forces have also always been regarded as of a mechanical nature. It has been shown by the experiments recorded in the present paper that the total cohesive force of any gel is influenced both by its ratio of surface to mass (thickness) and by the original concentration of the jelly at setting. With dried gelatin the cohesion has been further raised by the fact of drying.

A fact which emerges clearly from a review of the experimental data given in this paper is that under most circumstances the resistance of any gel to swelling is directly proportional to the amount of gelatin present in unit volume. (An apparent exception to this generalisation is found in freshly set jellies containing less than 10 % of gelatin, which eliminate water, *i.e.* undergo actual shrinkage. Equilibrium in water differs, however, from equilibrium in solutions of electrolytes, since neither osmotic forces nor hydration due to

salt action are factors in the final equilibrium.) It is obvious, therefore, that there is some definite structure in the gel which is imposing a restraint on swelling, and that this force of inhibition becomes increasingly greater, the greater the concentration of gelatin at the moment of setting. It is not unreasonable to suppose that this force acting against the swelling forces is due to a tangle of long threadlike micellae formed in the gel, and the purely mechanical nature of the cohesive forces is confirmed by the fact that thin sheets of gelatin offer less resistance to swelling than thick ones.

The absorption of water by gelatin is, therefore, affected, not only by the properties of the solution, but also by those of the gelatin gels. Three independent factors are to be attributed to the gels themselves: (1) a tendency for all gels in the presence of water to come to an equilibrium at 10 % concentration of gelatin; (2) an elastic resistance to swelling, which is purely mechanical and can be attributed to the forces required to move the entangled, threadlike micellae apart, and which falls off as the interstitial spaces between the micellae increase in volume; (3) in dry gelatin only, an additional effect which is to be attributed to some re-organisation occurring during drying.

Summary.

1. The absorption of water under varying conditions by discs of gelatin is influenced by the thickness of the disc. The swelling of both dried and undried gelatin gels in water or salt solutions is not greatly influenced by the thickness of the gel; in acid or alkaline solutions swelling is a negative function of the logarithm of the thickness.

2. The absorption of water by gelatin is, under all conditions, an inverse logarithmic function of the original concentration at which the jelly was set. This is most clearly shown in the undried jellies.

3. The amount of water absorbed by gelatin from water, solutions of salts, acids or alkalis is a function of the square of the temperature (degrees centigrade), as long as solution of the gel itself is inappreciable.

4. The influence of the original concentration of the gel depends on the amount of water in the system, irrespective of whether the water has penetrated into the jelly through the direct action of temperature, p_H value of the solution or the nature of the salts present. The effect of the original concentration is, therefore, purely mechanical in nature.

5. At low temperatures swollen gels in equilibrium with water tend to approximate to a concentration of 10 %, regardless of the original concentration of the gel.

6. In similar conditions gels which have been dried contain, at equilibrium, less water than those which have not been dried.

REFERENCES.

Gortner and Hoffmann (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 252, 257.
Hofmeister (1890). *Arch. exp. Path. Pharm.* **27**, 395.
Jordan-Lloyd (1920). *Biochem. J.* **14**, 147.
—— (1930). *Biochem. J.* **24**, 1460.
—— and Pleass (1927). *Biochem. J.* **21**, 1352.
—— —— (1928). *Biochem. J.* **22**, 1007.
Kunitz (1928). *J. Gen. Physiol.* **12**, 289.
Northrop (1927). *J. Gen. Physiol.* **10**, 893.
—— and Kunitz (1927). *J. Gen. Physiol.* **10**, 905.
Pauli (1897). *Arch. Physiol.* **67**, 219.
Pleass (1929). *Biochem. J.* **23**, 358.
—— (1930). *Biochem. J.* **24**, 1472.
Procter (1911). *Koll. Chem. Beihefte*, **2**, 243.
—— (1914). *J. Chem. Soc.* **105**, 313.
Smith (1919). *J. Amer. Chem. Soc.* **41**, 135.

CLXXII. THE EFFECTS OF CYANIDE AND SOME OTHER SALTS ON FERMENTATION BY YEAST PREPARATIONS.

By LAVINIA MARY BROWN PATTERSON[1].

From the Biochemical Department, The Lister Institute, London.

(*Received August 13th, 1931.*)

Particular interest is attached to the effect of HCN and cyanides on alcoholic fermentation because it is possible that one or other of the component reactions bringing about fermentation might be catalysed by metals and hence be specifically inhibited by cyanide.

Complete inhibition of fermentation with yeast juice by 1·2 % HCN (approx. $M/2$) was observed by Buchner, Buchner and Hahn [1903] but the minimum effective concentration had not been determined.

The effect of cyanides on the rate of hexosediphosphate formation by maceration extract and dried yeast at p_H 8·4 was examined by Neuberg and Kobel [1925]. They found that the reaction was delayed but finally went to completion, the p_H changing to about 6·8. Provided the p_H was not allowed to fall during the reaction, however, there was no observed effect with dried yeast. They were unable to give a definite explanation of the phenomenon.

Further, Neuberg and Perlman [1925] showed that 0·01–0·001 M cyanide slowed down the production of alcohol from sugar by maceration extract and dried yeast and in freshly fermenting yeast preparations completely inhibited it.

Warburg [1925] also investigated the effect of cyanide on alcoholic fermentation to decide whether it acted as a narcotic or by virtue of a chemical reaction. He found with maceration extract and living yeast that 0·01 N HCN diminished the rate of fermentation by 90–50 %, whereas with acetonitrile, a typical weak narcotic, a concentration of $2M$ was necessary to bring about a similar effect. He concluded that HCN was not acting as a narcotic.

All these authors found that the concentrations required to bring about these effects were greater than those required to bring about partial or complete inhibition of reactions catalysed by metals, which are of the order of 0·001–0·0001 M.

The yeast preparations, zymin and dried yeast, when mixed with a solution of either glucose or fructose do not begin to ferment immediately. The length of this delay during which there is no production of carbon dioxide varies with the volume of the mixture [Harden and Young, 1911] and is termed

[1] Part of a thesis approved for the Degree of M.Sc. in the University of London.

the induction period. It has now been found that the addition of KCN to the mixture lengthens this period; increasing concentrations of KCN producing a progressively longer induction period until, with sufficiently great concentrations, no fermentation occurs.

Experimental.

Effect of cyanide.

In determining the effect of KCN on the fermentation of glucose by yeast preparations, it was found advisable, because of the gradual removal of HCN gas from the system with the evolution of carbon dioxide, to use the apparatus of Slator [1906], in which fermentation is carried out at almost constant volume and at a reduced pressure.

Estimation of the cyanide content of three similar fermentation mixtures, (*a*) immediately after mixing, (*b*) and (*c*) after fermentation, yielded respectively 96, 89 and 86 % of the KCN added, so that the KCN does not suffer any serious loss.

A uniform method of mixing was adopted. The required volume of water was added to the glucose and yeast preparation and the whole saturated with CO_2. To this KCN was added, and the bottles were connected to the manometer with the taps shut off and then shaken; this ensured saturation with CO_2 without loss of HCN. The bottles were then placed in a thermostat at 30° and allowed to remain for 5 minutes until temperature equilibrium was attained. The pressure was then reduced and after shaking two or three times during a period of 5 minutes the initial reading was taken; the bottles were then shaken and read every 5 minutes. The period elapsing between the time of placing the bottles in the bath and the time when the rate of production of carbon dioxide became equal to the final steady rate was taken as the induction period.

The p_H of the mixture was not seriously affected by the addition of the concentrations of KCN used in these experiments and varied between 5·6 and 6·0, determined by the capillator method.

Fermentation. In fermentation mixtures with zymin, dried yeast and maceration extract, it was found that the presence of cyanide after lengthening the induction period

(*a*) slowed down the rate of attainment of the maximum rate in the presence of phosphates;

(*b*) did not affect the maximum rate attained;

(*c*) lowered the basal rate of fermentation (*e.g.* by 18 % with 0·006 *M* KCN; by 36 % with 0·01 *M* KCN) see Fig. 1.

In fermentations carried out with yeast juice, cyanide produced an induction period which was otherwise absent. This delay was followed by an enhanced rate falling to a basal rate, which was lower than in similar mixtures without cyanide.

These results show that cyanide chiefly affects the mechanism for the liberation of phosphate from hexosephosphate on which the basal rate depends, whilst the processes which occur in presence of free phosphate are unaffected.

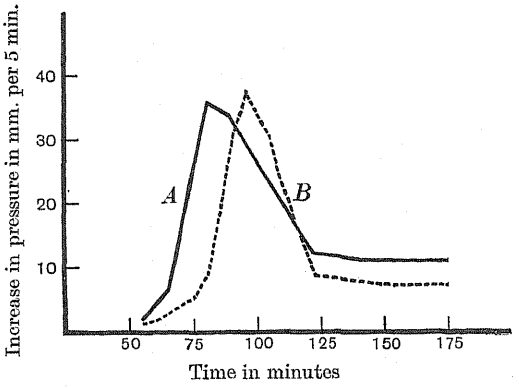


Fig. 1. *A* = Fermentation of control mixture after 65 mins. induction period.
B = Fermentation of mixture containing 0·008 *M* KCN after 225 mins. induction period.

Induction period. Zymin (yeast treated with acetone). Fermentation with 2 g. zymin and 2 g. glucose in 15 cc. volume showed a well-marked induction period. The addition of 0·0007 *M* KCN was ineffective, but from concentration 0·006 *M* KCN upwards the induction period was prolonged to an extent varying with the concentration (Table I, Exp. I, 1, 2, 5; 8, 9). As described by Harden and Macfarlane [1928] the addition of specific concentrations of

Table I.

No. of exp.	Yeast preparation	KCN *M*	K_2HPO_4 *M*	Potassium acetate	Potassium hexose-diphosphate	Induction period, minutes
I 1	Zymin (Z 11)	—	—	—	—	35
2	,,	0·008	—	—	—	55
3	,,	—	0·02	—	—	15
4	,,	0·008	0·02	—	—	35
5	,,	0·012	—	—	—	60
6	,,	—	—	0·2	—	10
7	,,	0·012	—	0·2	—	25
8	(Z 12)	—	—	—	—	25
9	,,	0·033	—	—	—	120
10	,,	—	—	—	0·005	10
11	,,	0·033	—	—	0·005	40

either K_2HPO_4, potassium acetate or potassium hexosediphosphate to a mixture of zymin and glucose solution shortened the induction period. In the presence of KCN and one of these salts the induction period was intermediate between that produced by KCN alone and the shortened one due to the salt action (Table I, Exp. I, 2, 3, 4; 5, 6, 7; 9, 10, 11).

Autofermentation. The autofermentation of zymin was also delayed in the presence of cyanide. 2 g. zymin (Z 14) in 30 cc. water commenced to ferment in 60 minutes but was delayed 25 minutes by 0·01 *M* KCN. With 2 g. zymin

(Z 18) in 20 cc. water the delay was increased from 40 to 55 minutes by the addition of 0·013 *M* KCN.

Dried yeast. This preparation behaved in a similar manner to zymin. 2 g. dried yeast and 2 g. glucose in 15 cc. volume showed an induction period of 45 minutes and this was progressively increased by the addition of KCN, being *e.g.* 75 minutes with 0·017 *M* and 100 minutes with 0·033 *M* KCN. Salts had the same effect on the induction period as with zymin.

Maceration extract (Table II). This shows an induction period [Lebedev, 1912] which to some extent resembles that shown by zymin, in as much as it is abolished by hexosediphosphate [Meyerhof, 1918]. The effect of KCN on this was investigated in the same manner as with zymin. Two methods of preparation of maceration extract were employed and the activities of the two solutions were found to differ. Using a dried yeast prepared from a Dutch baker's yeast Lebedev's [1911] method gave a less active solution than the more recent method of Hägglund and Rosenqvist [1927], according to which maceration was carried out in three parts of 0·15 *M* K_2HPO_4 or Na_2HPO_4 solution for 4 hours. This latter preparation had a shorter induction period than the former. The experimental mixtures consisted of 15 cc. maceration

Table II.

Maceration extract	Hexose-diphos-phate *M*	KCN *M*	K ace-tate *M*	Na_2HAsO_4 *M*	K_2SO_4 *M*	KCl *M*	Induction period minutes
Dy. 7 in water	—	—	—	—	—	—	No fermentation
,,	—	0·005	—	—	—	—	,,
,,	0·001	—	—	—	—	—	20
,,	0·011	0·005	—	—	—	—	145
,,	0·011	0·009	—	—	—	—	260
Dy. 9 in water	—	—	—	—	—	—	70
,,	0·005	—	—	—	—	—	15
,,	—	—	0·1	—	—	—	148
,,	0·005	—	0·1	—	—	—	30
,,	—	—	—	0·0025	—	—	100
,,	0·005	—	—	0·0025	—	—	30
,,	—	—	—	—	0·033	—	110
,,	—	—	—	—	—	0·075	No fermentation
,,	0·005	—	—	—	—	0·075	26
Dy. 11 in phos-phate solution	—	—	—	—	—	—	45
,,	—	0·015	—	—	—	—	No fermentation
,,	0·0033	—	—	—	—	—	15
,,	0·0033	0·015	—	—	—	—	100

extract and 2 g. glucose. The addition of KCN (0·005–0·015 *M*) to the mixture inhibited fermentation, which was not observed during 20 hours. When, however, hexosediphosphate was added at the same time as the cyanide fermentation took place, the induction period being always greater than in the presence of hexosediphosphate alone. The presence of Na_2HAsO_4 which prolongs the induction period with zymin [Harden and Macfarlane, 1928] also prolongs it with maceration extract and to some extent counteracts the shortening effect of hexosediphosphate. Such salts as KCl, K_2SO_4 and potassium acetate, which accelerate the onset of fermentation with zymin, delay

the onset with maceration extract both alone and in presence of hexosediphosphate.

By substituting sucrose for glucose Meyerhof [1918] found that the induction period of maceration extract was somewhat reduced. With the preparations used in the present experiments this only happened in the more active extracts macerated in phosphate solution, the induction period being shortened by 20 minutes.

It appears that the salts which diminish the induction period of zymin and dried yeast prolong it with maceration extract. Hexosediphosphate is the one exception to this, being the only substance tested which accelerated the onset of fermentation with maceration extract.

Yeast juice (Table III). Yeast juice together with glucose shows no induction period, but the addition of KCN of sufficient concentration produces

Table III.

Yeast juice	KCN *M*	Induction period, minutes
In all cases	—	0
Preparation No. 3	0·075	50
,, ,, 11	0·075	20
,, ,, 5	0·075	35
,, ,, 5	0·10	No fermentation
,, ,, 6	0·075	10
,, ,, 6	0·10	25
,, ,, 14	0·13	55

one. The length of the induction period varies with the concentration of KCN and with each sample of juice.

Changes in the form of combination of phosphorus during the induction period of yeast juice and maceration extract.

Yeast juice. To investigate any change in the form of combination of phosphorus that might be taking place during the induction period produced by the presence of KCN, flasks containing similar mixtures were placed in the thermostat and removed one at a time at intervals during the induction period. The action was stopped immediately by the addition of trichloroacetic acid up to 3 % and the phosphate estimations were carried out in the filtrate by the modified Briggs method (Table IV). The mixture consisted of 15 cc. yeast juice and 2 g. glucose (Exp. IV, 1–6).

During a short induction period produced by the presence of cyanide the acid-soluble organic phosphorus increased in amount at the expense of the inorganic phosphorus as esterification took place.

Exp. IV, 7–13. On the other hand, during the early part of a long induction period produced by KCN, hydrolytic action was observed, the acid-soluble inorganic phosphorus increasing in amount at the expense of the organic phosphorus. Esterification, indicated by increase in acid-soluble organic phosphorus, took place towards the end of the induction period.

During the induction period the total acid-soluble phosphorus remained unaltered.

Exp. IV, 2, 6, 8, 13. After fermentation had proceeded for some time and a steady rate had been established, the acid-soluble inorganic phosphorus was lower in the presence of cyanide than in its absence. At the same time a depressed basal rate was observed.

Maceration extract. In maceration extract the acid-soluble total phosphorus is practically all present as inorganic phosphorus.

Table IV, Exp. IV, 14, 15, 16. A mixture of 10 cc. of maceration extract made from Dy. 11, with 2 g. glucose in 15 cc. volume fermented after 45 minutes. During this time the total acid-soluble phosphorus (60·0 mg.) remained unchanged and the inorganic phosphorus (58·95 mg.) slowly decreased.

Table IV.

No. of exp.	Yeast preparation	Time after mixing (mins.)	KCN *M*	Hexose-diphos-phate *M*	Acid-soluble phosphorus: Total mg.	Acid-soluble phosphorus: Inor-ganic mg.	Acid-soluble phosphorus: Organic mg.	Fermentation commenced
IV 1	Yeast juice 13	0	—	—	76·91	23·51	53·40	Immediately
2	,,	70	—	—	,,	1·20	75·71	—
3	,,	0	0·05	—	,,	23·51	53·40	—
4	,,	10	,,	—	,,	19·52	57·39	—
5	,,	15	,,	—	,,	17·92	58·99	After 15 mins.
6	,,	85	,,	—	,,	1·11	75·80	—
7	Yeast juice 14	0	—	—	75·63	9·21	66·42	Immediately
8	,,	60	—	—	,,	1·92	73·71	—
9	,,	0	0·13	—	,,	9·21	66·42	—
10	,,	25	,,	—	,,	13·22	52·41	—
11	,,	35	,,	—	,,	15·43	60·20	—
12	,,	50	,,	—	,,	9·46	66·17	After 50 mins.
13	,,	115	,,	—	,,	1·52	74·11	—
14	Maceration extract	0	—	—	60·00	58·95	1·05	—
15	(Dy. 11)	20	—	—	,,	56·40	3·60	—
16	,,	40	—	—	,,	53·10	6·90	After 45 mins.
17	,,	0	0·015	—	58·80	58·80	—	—
18	,,	20	,,	—	,,	57·75	1·05	—
19	,,	40	,,	—	,,	57·30	1·50	—
20	,,	70	,,	—	,,	57·15	1·65	—
21	,,	130	,,	—	,,	55·95	2·85	No fermentation
22	,,	0	—	0·0033	64·65	59·10	5·55	—
23	,,	7	—	,,	,,	59·55	5·10	—
24	,,	12	—	,,	,,	55·95	8·70	After 15 mins.
25	,,	0	0·015	0·0033	63·75	58·80	4·95	—
26	,,	20	,,	,,	,,	59·10	4·65	—
27	,,	40	,,	,,	,,	58·80	4·95	—
28	,,	70	,,	,,	,,	55·95	7·80	—
29	,,	90	,,	,,	,,	55·65	8·10	—
30	,,	100	,,	,,	,,	55·50	8·25	After 100 mins.

Exp. IV, 17–30. Fermentation was completely inhibited in such a mixture by 0·015 *M* KCN. In 130 minutes the acid-soluble inorganic phosphorus decreased by 2·85 mg. The total phosphorus remained unaltered. The addition of 0·0033 *M* potassium hexosediphosphate shortened the induction period considerably from 45 to 15 minutes in the control without cyanide, during which time there was a small amount of esterification shown by a decrease of 3·15 mg. in the acid-soluble inorganic phosphorus. A similar decrease

(3·3 mg.) took place during the 100 minutes' induction period of a mixture containing 0·015 *M* KCN as well as 0·0033 *M* potassium hexosediphosphate. In all these cases the total acid-soluble phosphorus remained constant and no change occurred except a slight esterification towards the end of the induction period.

The induction period of zymin.

In an attempt to arrive at an explanation of the cause of the induction period before fermentation with zymin, the required amount of zymin was ground up in a 12-hour old glucose solution and the desired quantities of the mixture were pipetted into test-tubes which were placed in a thermostat. Determinations of the optical rotation, reducing power and phosphates were made in the trichloroacetic acid filtrates at zero time and subsequently at intervals during the induction period. The reducing power as glucose of the mixture was determined by the Hagedorn-Jensen method (Table V). During the early part of the induction period of 100 minutes of a mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in a total volume of 60 cc. there were only

Table V.

Mixture	Time after mixing	Optical rotation α_{5461}	Reducing power as glucose mg./1 cc.	Fermentation commenced
3 g. Z 18 + 48 cc. glucose solution in total volume 60 cc.	0	+2·90	44·4	—
	70	+2·81	45·0	—
	80	+2·81	44·6	—
	90	+2·75	42·6	After
	100	+2·68	42·4	100 mins.

slight changes observed in either optical rotation or reducing power. As fermentation commenced these values slowly decreased, as might be expected. In mixtures with 0·13 *M* and 0·2 *M* potassium acetate, which shorten the induction period, and with 0·015 *M* KCN or 0·0067 *M* Na_2HAsO_4 which lengthen the induction period, and with fructose substituted for glucose the same order of changes occurred.

Phosphorus estimations. As before, these estimations were carried out according to the modified Briggs method. On investigating the changes occurring during the induction period when no carbon dioxide was given off, it became evident that the form of combination of the phosphorus altered in an arresting manner [see also Macfarlane, 1931].

Table VI, Exp. VI, 1–7. In the control mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in a total volume of 60 cc. there was an increase in the total acid-soluble phosphorus from 33·00 mg. to 53·66 mg. during the 100 minutes' induction period. During the early part of the induction period there was an almost corresponding increase in the acid-soluble inorganic phosphorus, followed by a decrease towards the time of commencement of fermentation. The acid-soluble organic phosphorus remained almost constant

Table VI.

No. of exp.	Mixture	Time after mixing minutes	Acid-soluble phosphorus			Fermentation commenced
			Total P mg.	Inorganic P mg.	Organic P mg.	
VI '1	3 g. Z 18 + 48 cc. glucose	0	33·00	10·63	22·37	
2	solution in total volume	20	39·60	18·74	20·86	
3	60 cc.	40	44·63	23·70	20·98	
4		70	47·85	19·47	28·38	
5		80	50·95	13·73	37·22	
6		90	52·80	5·87	46·93	After
7		100	53·66	4·69	48·97	100 mins.
8	3 g. Z 18 + 48 cc. glucose	0	38·35	10·97	27·38	
9	solution + 0·13 *M* potas-	25	46·79	16·90	29·89	
10	sium acetate in total	35	49·24	5·21	44·03	After
11	volume 60 cc.	40	49·96	5·08	44·88	45 mins.
12	3 g. Z 18 + 48 cc. glucose	0	46·79	11·15	35·64	
13	solution + 0·2 *M* potas-	7	49·43	13·00	36·43	
14	sium acetate in total	14	50·36	6·14	44·22	
15	volume 60 cc.	21	50·56	3·76	46·80	After
16		28	50·82	3·78	47·04	25 mins.
17	3 g. Z 18 + 48 cc. glucose	0	34·91	10·63	24·28	
18	solution + 0·01 *M* KCN	20	38·74	14·32	24·42	
19	in total volume 60 cc.	40	41·38	21·12	20·26	
20		70	46·46	26·93	19·53	
21		100	50·86	32·57	18·29	
22		130	52·07	38·48	13·59	
23		150	52·07	38·15	13·82	
24		170	52·20	35·64	16·56	
25		190	52·58	28·18	24·40	
26		210	52·67	26·80	25·87	After
27		230	52·80	13·46	39·34	230 mins.
28	3 g. Z 18 + 24 cc. glucose	0	40·25	11·02	29·23	
29	solution in total volume	25	45·46	11·88	33·58	
30	30 cc.	35	49·39	7·70	41·69	After
31		40	50·29	7·09	43·20	40 mins.
32	3 g. Z 18 + 24 cc. glucose	0	44·03	11·48	32·55	
33	solution + 0·015 *M* KCN	25	44·28	21·17	23·11	
34	in total volume 30 cc.	35	46·55	23·87	22·68	
35		55	48·78	28·55	20·23	
36		70	52·20	28·12	24·08	
37		80	52·20	15·52	36·68	After
38		100	52·34	7·20	45·14	100 mins.

Z 18 = Zymin No. 18

for the first part of the induction period and then rapidly increased as esterification took place.

Exp. VI, 8–11. In the 45 minutes' induction period in the presence of 0·13 *M* potassium acetate in a similar mixture the total acid-soluble phosphorus increased to almost the same value as in 100 minutes in the control. The absolute increase was, however, less because the total acid-soluble phosphorus at mixing was higher in the presence of acetate than in its absence. As in the control, the acid-soluble inorganic phosphorus at first increased and then later decreased. The organic phosphorus remained almost constant for the first 25 minutes and then increased.

Exp. VI, 12–16. In the presence of 0·2 *M* potassium acetate the induction period of this mixture was further shortened to 25 minutes and when fermentation began the total acid-soluble phosphorus was again approximately the

same; the absolute increase being again less owing to the higher original total acid-soluble phosphorus. Both the inorganic and organic phosphorus behaved as in the former case with acetate present.

If the changes observed in the concentration and rate of extraction of the total acid-soluble phosphorus in zymin bear any relation to the length of the induction period it might be expected that the increase in the rate of extraction of phosphorus coincident with a shorter induction period, which is shown to take place in presence of acetate, would be reversed with cyanide, and this was found to be the case.

Exp. VI, 17–27. The presence of 0·01 *M* KCN in a mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in 60 cc. volume prolonged the induction period by 130 minutes. The total acid-soluble phosphorus values at mixing and at the onset of fermentation were almost the same as in the control. The absolute increase was therefore identical, but the period required for its production was almost $2\frac{1}{2}$ times as long (see Fig. 2).

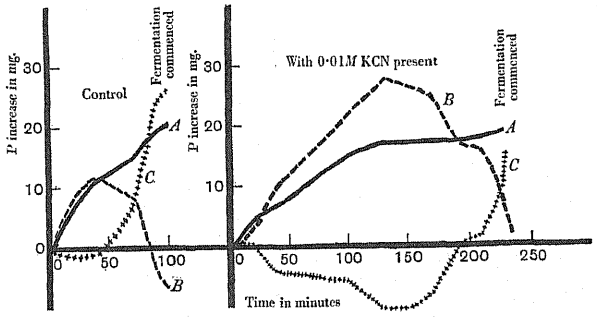


Fig. 2. *A* = acid-soluble total phosphorus. *B* = acid-soluble inorganic phosphorus. *C* = acid-soluble organic phosphorus.

Exp. VI, 28–38. This relation was again shown in two mixtures of half the total volume. 3 g. zymin (Z 18) + 24 cc. glucose solution in 30 cc. volume, one with 0·015 *M* KCN and one without. The absolute increase of total acid-soluble phosphorus in the two mixtures was about the same, but its production required 60 minutes longer in the mixture containing cyanide.

As shown in the experiments with and without potassium acetate present, the amount of increase of acid-soluble inorganic phosphorus during the first part of the induction period was approximately the same as that of the total phosphorus. The extra phosphorus derived from the zymin, which was estimated as acid-soluble inorganic phosphorus may have been present in the zymin in that form or as an ester which was hydrolysed; there was, however, no direct evidence of this hydrolysis. On the other hand, in the presence of cyanide the increase in inorganic phosphate was greater than the increase in total phosphorus, which indicates that hydrolysis at the expense of the organic phosphorus, which gradually decreased, was occurring. Esterification occurred towards the end of the induction period as in other cases (see Fig. 2).

In this present set of experiments the only evidence of hydrolysis of organic phosphorus throughout the early part of the induction period of zymin fermentation was obtained in the experiments with cyanide.

This observation that fermentation takes place when an approximately constant concentration of total acid-soluble phosphorus derived from the zymin is reached, throws some light on the nature of the effect of salts. In all the experiments with 3 g. zymin (Z 18) in 60 cc. volume both in the presence and absence of added salts, fermentation began when the concentration of total acid-soluble phosphorus was between 49·0 mg. and 54·0 mg. At mixing the acid-soluble total phosphate was 33·00 mg. in the control, 38·35 mg. in the presence of 0·13 *M* potassium acetate and 46·79 mg. with 0·2 *M* potassium acetate. It was thus seen that of the mixtures in which the same concentration of total phosphorus was reached before fermentation commenced, those in which the total phosphorus at mixing was higher had the shorter induction periods.

In another mixture of 3 g. zymin (Z 18) in 30 cc. volume, *i.e.* half the volume in the former experiments, the total at mixing was 40·25 mg. increasing to 50·29 mg. in 40 minutes when fermentation began. The length of this induction period and the total acid-soluble phosphorus both at mixing and when fermentation began are comparable with those of the mixture in double the volume containing 0·13 *M* potassium acetate. The presence of this salt to some extent counteracts the effect of dilution.

A comparison of the two control mixtures, one in 60 cc. volume and the other in 30 cc., further illustrates this point. At mixing, the total acid-soluble phosphorus of the more dilute mixture was 33·00 mg., this was lower than that of the mixture in 30 cc., which was 40·25 mg. They both began to ferment when the total phosphorus was approximately the same (50·29 mg. in 30 cc. and 53·66 mg. in 60 cc.), but the time taken to arrive at this concentration was 60 minutes longer in the more dilute mixture. Hence dilution retarded the accumulation of phosphate from zymin and increased the induction period.

There still remained a certain amount of phosphorus in the zymin, even after 3 hours' incubation, for the highest value of total acid-soluble phosphorus obtained was never as high as the absolute total phosphorus found. Thus the total phosphorus of 1 g. zymin (Z 18) was 21·0 mg. and the highest total acid-soluble found was 17·5 mg.

Inorganic phosphorus during the basal rate.

It has already been shown that the basal rate of fermentation of sugar solution with zymin was reduced in the presence of cyanide. The acid-soluble inorganic phosphorus of a mixture of 2 g. zymin (Z 14) + 2 g. glucose in 30 cc. volume fermenting at a steady rate was 1·70 mg. By the presence of 0·007 *M* KCN this was reduced to 1·40 mg. and to 1·26 mg. by the presence of 0·01 *M* KCN. These results again show that the presence of cyanide has

interfered with the mechanism by which phosphate is obtained from hexose-diphosphate.

Discussion.

It is inferred, partly from the negative results obtained with regard to the optical rotation and reducing powers of the mixtures and partly from the positive evidence of changes in the form of combination of the phosphorus, that the duration of the induction period of fermentation mixtures with zymin is dependent on changes taking place in the zymin. It is uncertain whether the actual release of phosphate or whether its condition of availability is the necessary factor to bring about the commencement of fermentation. It is also conceivable that the liberation of phosphate may be accompanied by other secondary changes in the zymin by which another substance or group of substances directly influential in bringing about fermentation is made available. At present it is obscure how the liberation of phosphate is hastened or retarded, but the question of an alteration of the permeability of the cell wall of the acetone yeast preparation arises. Potassium acetate which shortens the induction period increases the rate at which the available phosphate becomes acid-soluble. In the presence of cyanide, however, which increases the induction period, and decreases the rate of increase of acid-soluble phosphorus, fermentation does not begin when the concentration of total phosphorus is reached which is effective in the control or in the presence of potassium acetate. The cyanide evidently also exerts some other effect. The essential point does not seem to be the accumulation of organic phosphorus, as this is decreasing throughout nearly the whole of the induction period in the presence of KCN and its increase towards the end is probably the result of the fermentation and not the cause of it, there being always a lag between esterification and evolution of CO_2 [Boyland, 1930].

Cyanide and arsenate.

It having being definitely established that both cyanide and arsenate prolong the induction period of zymin and glucose mixtures, it seemed probable that by introducing these salts independently and together into a mixture some light might be thrown on the nature of their separate actions (see Fig. 3). A mixture of 2 g. zymin (Z 18) with 2 g. glucose in 15 cc. had an induction period of 30 minutes. This was prolonged to about 70 minutes in presence of either 0·013 *M* KCN or of 0·0067 *M* Na_2HAsO_4 (called concentration 1). On doubling and trebling these concentrations (concentrations 2 and 3) almost proportional increases in the induction period were observed with KCN, whereas with arsenate the increases were considerably less (Fig. 3, Curves A and B). It seems, therefore, that the mode of action of the two substances is quite different.

In attempting to investigate this point further the two salts were added together to the mixture and the time effects observed. By adding at the same time the original concentrations (concentration 1) of each salt, the

increase in the induction period (curve *C*) was intermediate between those obtained with concentration 2 of the salts separately, and nearer to the time

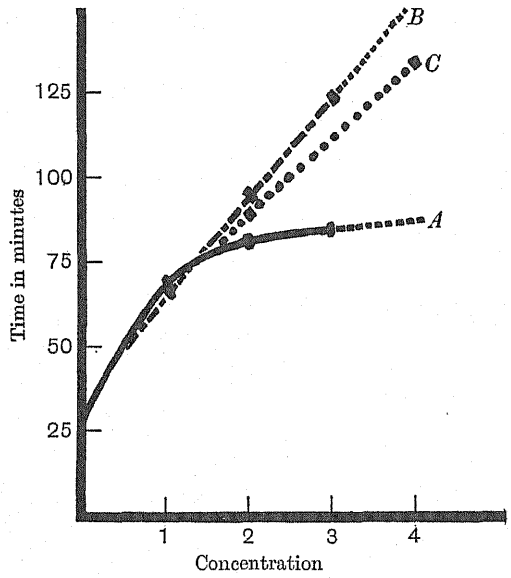


Fig. 3. *A* = Induction period due to Na_2HAsO_4. *B* = Induction period due to KCN. *C* = Induction period due to Na_2HAsO_4 and KCN.

due to the cyanide effect. The addition of 0·026 *M* KCN and 0·0134 *M* Na_2HAsO_4 (concentration 2) together to a mixture produced an induction period, which lies between those which would be expected from the curves of concentration 4 of KCN and Na_2HAsO_4 added separately. The effect was thus found to be more than that of either salt alone but less than the sum of those produced by the two salts alone. At the same time it was more than that produced by the double concentration of arsenate, but less than that due to the double concentration of cyanide. This suggests that interference takes place between the actions of the two substances possibly because they may affect reactions dependent to a certain extent on one another.

Methylglyoxal.

It is supposed that methylglyoxal occurs as an intermediate product of alcoholic fermentation. If this substance is formed towards the end of the induction period as suggested by Boyland [1930], by including it in the original fermentation mixture a shortening of the induction period might be brought about. This has been found to occur in the present experiments with zymin. As the sample of methylglyoxal hydrate[1] used was acid in reaction (p_H 1·6) before each experiment the p_H was adjusted to 6·0–6·2 with Na_2CO_3.

[1] I am indebted to Prof. R. Robinson for this specimen of methylglyoxal.

Table VII.

No. of exp.	Mixture	Methyl-glyoxal *M*	Induction period, minutes	Reduction of Phosphate rate %	Reduction of Basal rate %
VII 1	2 g. Z 18 + 2 g. glucose in	—	60	—	—
2	20 cc. volume	0·01	60	—	—
3		0·01*	—	—	—
4		0·02	10	45	40
5		0·02*	—	25	25
6		0·03	15	60	60
7		0·03*	—	35	38
8		0·05	20	Complete	52
9		0·05*	—	52	52
10		0·1	30	Complete	60
11		0·1*	—	60	60
12	2 g. Z 18 + 16 cc. water	—	25	—	—
13		0·05	15	55	60
14	2 g. Z 18 + 26 cc. water	—	70	—	—
15		0·02*	—	30	33
16		0·03*	—	54	55

* Denotes addition at the end of the normal induction period.

Table VII, Exp. VII, 1, 2, 3, 4. The mixture used was 2 g. zymin (Z 18) and 2 g. glucose in 20 cc., which had an induction period of 60 minutes. The concentration of methylglyoxal which had the maximum time effect and shortened the induction period to 10 minutes was 0·02 *M*. After 65 minutes of fermentation at a reduced rate a maximum rate was reached, which was less than in the control without methylglyoxal. The basal rate was reduced by 40 % in the presence of methylglyoxal.

Exp. VII, 6, 8, 10. Concentrations of 0·03 *M*, 0·05 *M* and 0·1 *M* shortened the induction period and increasingly reduced the basal rate up to 60 % in the case of 0·1 *M*. No rise to a maximum rate occurred in the presence of either 0·05 *M* or 0·1 *M* methylglyoxal.

Exp. VII, 5, 7, 9, 11. To determine the effect on fermentation, similar concentrations to the above were added at the onset of fermentation (*i.e.* after the normal induction period of 60 minutes of the mixture). The evolution of CO_2 decreased with increasing concentration of methylglyoxal, the basal rate being reduced 40 % with 0·02 *M* and 60 % with 0·1 *M* methylglyoxal.

Table VIII. On addition of K_2HPO_4, in optimum concentration (0·09 *M*),

Table VIII.

Mixture	K_2HPO_4 *M*	Methyl-glyoxal *M*	Rate of CO_2 production: Highest	Basal	Phosphate rate (cc. per 5 minutes)	Percentage reduction: Phosphate rate	Basal rate
2 g. Z 18 + 2 g. glucose in 15 cc. volume	0·06	—	12·8	3·2	9·6	—	—
	0·06	0·035	10·9	2·3	8·6	10·4	28·1
	0·09	—	13·6	3·1	10·5	—	—
	0·09	0·02	12·5	2·9	9·6	8·5	6·4
	0·09	0·035	11·5	2·7	8·8	16·1	12·6
	0·09	0·05	9·4	2·1	7·3	30·4	32·2
	0·15	—	13·1	3·4	9·7	—	—
	0·15	0·05	9·0	2·1	7·9	18·5	38·2

together with methylglyoxal in various concentrations at the onset of fermentation, a lower maximum phosphate rate and basal rate were observed than in the absence of methylglyoxal. With 0·02 *M* methylglyoxal the maximum rate was 8·5 % and with 0·05 *M* 43·2 % less than the control, the basal rates of the same mixtures being 6·1 % and 32·2 % lower. In the presence of methylglyoxal the maximum rate was attained about 10 minutes sooner than in its absence.

Exp. VII, 12–16. The autofermentation of zymin (Z 18) in the presence of methylglyoxal showed the same inhibition. With 0·05 *M* methylglyoxal the induction period was shortened from 25 minutes to 15 minutes and the basal rate reduced by 60 %. At the onset of autofermentation the addition of 0·02 *M* and 0·03 *M* methylglyoxal reduced the maximum rates 30 % and 54 % respectively and the basal 33 % and 55 %. There was no indication of fermentation of methylglyoxal as there was a smaller evolution of carbon dioxide in its presence than in its absence.

It appears from these experiments that methylglyoxal exerts an inhibiting influence, either directly or indirectly, both on the process of esterification and on the mechanism which controls the basal rate. At the same time it shortens the induction period.

Summary.

1. In alcoholic fermentation by yeast preparations KCN (minimum concentration 0·006 *M*) slows down the attainment of a maximum rate in the presence of phosphate, without affecting the maximum rate attained. The basal rate of fermentation is lowered.

2. The induction period before the onset of fermentation by yeast preparations (zymin, dried yeast and maceration extract) is increased by cyanide (minimum concentration 0·005 *M*); with yeast juice, cyanide (minimum concentration 0·075 *M*) produces an induction period which is otherwise absent.

3. In the presence of substances such as potassium acetate, potassium hexosediphosphate and dipotassium hydrogen phosphate, which shorten the induction period of zymin and dried yeast, cyanide causes a prolongation of it to a smaller degree than when acting alone.

4. With yeast juice in the presence of cyanide, the total acid-soluble phosphorus remained constant, the organic phosphorus diminished and the inorganic phosphorus increased till near the end of the induction period.

5. With maceration extract the total phosphorus remained constant and there was no change except slight esterification towards the end of the induction period.

6. During the induction period of zymin under various conditions the total and inorganic acid-soluble phosphorus increased by the same amount while the organic phosphorus remained approximately constant till near the end of that period, when it increased as the inorganic phosphorus became esterified.

7. In the presence of cyanide during the first part of the induction period there was a greater increase of acid-soluble inorganic phosphorus than of total phosphorus. This was due to hydrolysis of the organic phosphorus.

8. The optical rotation and reducing power of the trichloroacetic acid filtrates from the fermentation mixtures only showed slight changes until the end of the induction period was approached.

9. In the presence of arsenate, which prolongs the induction period of zymin, cyanide further prolongs it, but the joint effect is less than the sum of the separate effects. The curve connecting increase of induction period with increase of concentration is different for each salt, indicating that their actions are of a different kind.

10. Methylglyoxal shortened the induction period of fermentation with zymin and reduced both the basal rate of fermentation and the rate of esterification.

I desire in conclusion to express my thanks to Prof. A. Harden for his constant advice and help in this work.

REFERENCES.

Boyland (1930). *Biochem. J.* **24**, 703.
Buchner, Buchner and Hahn (1903). Die Zymasegärung, 181. (München.)
Hägglund and Rosenqvist (1927). *Biochem. Z.* **180**, 61.
Harden and Macfarlane (1928). *Biochem. J.* **22**, 786.
Harden and Young. *See* Harden (1911). Alcoholic fermentation, p. 37. (Longmans, Green and Co., London.)
Lebedev (1911). *Compt. Rend. Acad. Sci.* **152**, 49.
—— (1912). *Ann. Inst. Past.* **26**, 8.
Macfarlane (1931). *Biochem. J.* **25**, 822.
Meyerhof (1918). *Z. physiol. Chem.* **102**, 185.
Neuberg and Kobel (1925). *Biochem. Z.* **155**, 499.
—— and Perlman (1925). *Biochem. Z.* **165**, 238.
Slator (1906). *J. Chem. Soc.* **89**, 128
Warburg (1925). *Biochem. Z.* **165**, 196.

CLXXIII. A SPECTROGRAPHIC ANALYSIS OF HUMAN TISSUES.

By JOSEPH HAROLD SHELDON and HUGH RAMAGE.

From the Royal Hospital, Wolverhampton, and Ridgemont, Carrow Hill, Norwich.

(*Received July 31st, 1931.*)

This paper describes the results of a spectroscopic examination of 195 specimens of different human tissues, both healthy and diseased. The object of the investigation was to determine whether any chemical elements were constantly present in the tissues in addition to those already recognised as being universal constituents of protoplasm (C, H, O, N, P, S, Na, K, Mg, Ca, Cl, Fe, Cu) and to discover what variations might occur both between different organs and between healthy and diseased tissues.

Preparation of the material.

A general description of the method has been published by one of us [Ramage, 1929]. Small pieces of the organ were removed and washed in distilled water to free them from blood as far as possible. They were then placed in a steam-oven and dried until of constant weight. In cases where the presence of fat made this difficult, the melted fat was removed by drying them on the ashless filter-paper used for dealing with fluid specimens. The resulting material was then ground to powder in an agate mortar, and a small amount of this powder was used for obtaining the spectrum. Except where it is otherwise stated, the amount of each specimen that was used was 0·05 g. This was rolled in a small piece of ashless filter-paper—weighing about 0·12 g.—cut from Munktell's Swedish paper No. 00 diameter 12·5 cm., and the whole was then burnt in an oxy-coal-gas flame before the slit of a quartz spectrograph, a quartz lens being used to focus the image of the flame on the slit. The spectrum of the flame itself has no metallic lines, but shows a number of lines in the ultra-violet due to water-vapour. The spectrum of the filter-paper shows very weak lines due to sodium, potassium, and calcium, but apart from these is devoid of metallic lines. The spectrograph employed was a Size C Hilger quartz spectrograph, and the photographic plates have been Ilford panchromatic coated on thin glass. Plates 5″ by 4″ suitably placed in the holder cover the region from the red to beyond λ2800, which is that required when no arc is used. In the case of fluids such as blood, 0·1 cc. was transferred to a piece of the filter-paper which had previously been rolled

from the curved edge, dried in the steam-oven, and burnt in front of the spectrograph in the same way as the solid specimens.

Standard solutions were prepared having the following percentage composition: (1) Na, 0·15; K, 0·20; Ca, 0·02; Li, 0·0004; Rb, 0·004; Mg and Fe, 0·05; Cu and Co, 0·005; Mn and Ni, 0·002; Cd, 0·10; P, 0·50; and (2) Sr, 0·002; Ag, 0·0025; and Pb, 0·05. The brilliance of the lines obtained on burning papers charged with the standard solutions naturally varies with the amount of solution used, and since it is possible to obtain the photographs of 16 spectra on the one photographic plate, four of these were devoted to the standard solutions, each in amounts of 0·02, 0·05, 0·1 and 0·2 cc. In this way, by matching the intensity of the lines of an element in a specimen with that in the different strengths of standard solution, the method can be put to a quantitative as well as a qualitative use.

Though figures are given throughout this paper, it is not desired to lay stress at present on the quantitative nature of the investigation, the original purpose of which was a qualitative survey of the metallic constituents of the various tissues. Only the four strengths of standard solution already mentioned were employed, and in consequence, where the brightness of the lines of a particular element is less than that shown by the element in the weakest strength of the standard solution, a "trace" of the element in question has usually been said to be present. In using the method further for quantitative purposes, either a more extensive range of variation of the strengths of standard solution could be employed, or the standard weight of specimen burnt (0·05 g. here) could be altered, so that the lines could be accurately matched.

Except in the case of blood, all the percentages given are in terms of the dry weight of the tissue. Where the term "trace" is used, the element in question is present in a percentage of less than the following, which are the percentages represented (when 0·05 g. of specimen is used) by lines matching those formed by the burning of 0·02 cc. of standard solution.

	%		%
Sodium	0·06	Manganese ...	0·0008
Potassium ...	0·08	Iron	0·02
Lithium	0·00016	Silver	0·001
Calcium	0·008	Strontium ...	0·0008
Rubidium ...	0·0016	Lead	0·02
Magnesium ...	0·02	Phosphorus ...	0·20
Copper	0·002		

By this method the following elements in addition to those mentioned above may be readily detected when present in 0·05 g. of a specimen: caesium, barium, cadmium, gallium, indium, thallium, bismuth, chromium, cobalt, nickel, ruthenium, rhodium, palladium and iridium. Other elements have been described as occurring in plant or animal tissues which are not included in this list, of which the more important are zinc, aluminium, tin and

titanium. These elements can be detected when an arc spectrum is employed, and in a recent paper by Zbinden [1930] were all described as occurring in human tissues. Zinc has been described by Bodansky [1921] as a normal constituent of the brain. The reason why these elements cannot be detected directly by the method employed here has already been given by Fox and Ramage [1931], *viz.* "that the thermal or electrical conditions of the flame are not sufficiently intense either (*a*) to reduce the oxides in adequate quantities or (*b*) when small quantities only are present, to excite the vibrations producing the lines with enough energy to photograph them, placed, as most of them are, in the further portion of the ultra-violet region of the spectrum, *i.e.* beyond wave-length 3100." The arc spectrum employed by Zbinden, making use of carbon electrodes, while more sensitive, introduces a risk of error due to impurities in the carbons *etc.*, which is not present in our method.

In view of the fact that the presence and distribution of sodium, calcium, potassium and magnesium through the body is already well known, these metals are not referred to except where they show some striking anomaly.

The cases from which tissues were taken for examination may be summarised as follows:

S. Male, age 77. Death from cerebral haemorrhage, due to arteriosclerosis. The heart was enlarged and adherent to the pericardium. The pericardial adhesions were calcified in places, and the mitral valve also showed calcified nodules.

F.M. Female, age 69. Death from cholangitis, following chronic inflammation of the gall-bladder.

K.T. Female, age 2. Death from acute broncho-pneumonia.

A.B. Male, age 35. Death from pneumonia following operation for a perforated duodenal ulcer.

G.E. Male, age 53. Squamous-celled carcinoma of the oesophagus.

N.C. Female, age 27. Death from acute tubercular broncho-pneumonia. Both lungs showed widespread tubercular deposits, without obvious caseation. The thyroid gland contained a small adenoma.

M.R. Female, age 7. Acute broncho-pneumonia.

M.L. Male, age unknown. Advanced chronic interstitial nephritis.

E.C. Female, age 5. Death from diphtheritic toxaemia.

G.C. Male, age 9 months. Acute broncho-pneumonia.

R.M. Male, age 61. Death from uraemia.

H. Male, age 25. Miliary tuberculosis.

Foetus D, 24 weeks development. This was a normal foetus, which was removed at operation by Mr Maslen Jones, owing to heart disease of the mother.

Foetus D (*a*), 24 weeks development. Abortion of unknown origin.

Foetus F, female, full-term development. Stillborn.

Foetus (10), foetus of 10 weeks' development. Abortion.

L. Female, age 20. Chronic lymphatic leukaemia.

J. Male, age 72. Advanced atrophic cirrhosis of the liver, which was probably alcoholic in origin.

E.B. Male, age 49. Death from tuberculosis with advanced silicosis of the lung.

P. Male, age unknown. Advanced silicosis of the lung.

L (2). Female, age unknown. Myeloid leukaemia, death occurring while 26 weeks pregnant.

Foetus L (2). The foetus of the above case.

B.P. Age 9 months. Death from miliary tuberculosis.

G.P. Owing to the difficulty of freeing the human tissues removed at *post-mortem* from all their contained blood, this was done in the case of a guinea-pig. The animal was killed by bleeding,

and immediately after death a cannula was inserted into the aorta and the animal transfused with distilled water. The organs, after being dissected out, were minced and again washed in glass-distilled water before being placed in the drying oven.

Description of the various tissues.

Table I. *Liver.*

Patient	Copper	Manganese	Rubidium	Silver
S.	Trace	0·0004	0·002	Trace
F.M.	0·0015	0·0004	0·002	Trace
A.B.	0·002	0·0005	0·003	Trace
G.E.	0·003	0·0008	0·004	Trace
N.C.	0·0015	Trace	0·0016	—
Foetus D	0·035	0·001	0·004	Merest trace
Foetus (10)	0·015	Trace	None	Merest trace
J.	0·02	0·0008	0·0016	—
E.C.	0·002	0·0008	0·0024	Trace
Foetus F	0·025	0·001	0·0016	Trace
Foetus D (*a*)	0·03	0·0004	0·002	—
G.C.	0·002	Trace	0·002	—
R.M.	0·02+	0·0008	0·004	—
Foetus L (2)	0·02+	Trace	0·0016	—
L (2)	0·002	0·0008	0·0016	Trace
B.P.	0·02	Trace	0·0024	—
G.P.	0·0075	0·002	0·004	—

Liver. The iron spectrum was rich in all the specimens examined, including that of the transfused guinea-pig. The lines of Na, K, Ca and Mg reveal no unusual features, except that the liver of the 10-weeks foetus was very high in Ca and very low in Mg and K. Copper was present throughout, and, in general, the lines indicate a higher concentration in this organ than in any other region of the body; *e.g.* in the transfused guinea-pig, 0·0075 % in the liver, as against slightly less than 0·002 % for the other organs of this animal that were examined. The most prominent feature of the distribution of copper in the liver as shown by Table I is the high Cu content of the foetal liver, the lowest amount, found in the 10-weeks specimen (0·015 %), being some three times as much as that of the average figure (0·0045 %) for the adult livers. This particular specimen was in an advanced state of autolysis when dissected out, and it was only possible to obtain 34 mg. of dry tissue for burning. The other four foetal specimens have an average of 0·0275 %. The difference is strikingly shown by the case of L (2) where the maternal liver had 0·002 % Cu and the foetal liver more than 0·02 % Cu. That there is considerable variability in the rate of dispersion of copper from the liver after birth is shown by the difference between the Cu contents of the livers of two 9 months old babies (G.C. 0·002 % and B.P. 0·02 %). The high Cu content of the foetal liver is probably connected with its blood-forming functions in this stage of life, since it has been shown by Waddell *et al.* [1928, 1929] that the presence of this element is necessary for the formation of haemoglobin.

Specimens J. and R.M. are of interest, since these were the only adult livers to give copper lines of an intensity comparable with those found in the

foetus. In the case of J. the liver was examined in the first place with the idea that it might contain an abnormal amount of manganese, in view of the facility with which cirrhosis of the liver can be produced in experimental animals by this metal. The manganese line was, however, weak, but the copper lines were very strong (0·02 %). Whether the presence of this large amount of copper bears any relation to the development of the disease is uncertain. Schonheimer and Oshima [1929] state that cirrhosis of the liver may occur with a normal amount of copper. The case of R.M. (uraemia) is of importance in the reverse connection, since the liver contained slightly more than 0·02 % of copper, while the organ itself, though large, was normal in appearance except for some fatty degeneration, and showed no sign of cirrhosis. Manganese was found in every liver, and in five instances the amount present was less than 0·0008 %. The organs of the baby G.C. who died at the age of 9 months from broncho-pneumonia, had, in general, a low content of copper and manganese, which raises the interesting speculation as to whether a deficiency of these metals plays any part in lowering resistance towards infection. The transfused guinea-pig gave the strongest spectrum (0·002 %) while among the human specimens foetus D and foetus F had the greatest amount (0·001 %). As with copper, the liver apparently has a greater need for manganese during foetal life than after birth. The amount of manganese was low in both foetus (10) and foetus D (*a*), but both of these died from unknown causes, while foetuses F and D were healthy.

Rubidium was present in every liver except that of the 10-weeks foetus. The lines were generally strong, though there were considerable individual variations, the greatest amount in the adults (0·004 %) being found in G.E. (carcinoma of the oesophagus), R.M. (uraemia) and in the liver of foetus D.

Traces of silver occurred in 9 of the 17 specimens of liver. A trace of lead was found in the case of G.E. and the liver of the 10-weeks foetus was unique in containing a trace of strontium. It is worthy of note that 3 of the 5 foetal livers contained traces of silver.

Gall-bladder and bile. Four specimens were examined. (Gall-bladders G.E. and foetus F, bile from foetus F, and both gall-bladder and bile were examined together from foetus D. The bile was dried before examination.)

The gall-bladder of G.E. had strong Ca lines, presumably the result of age. The Cu lines were strong in both gall-bladder and bile of foetus F, the gall-bladder containing 0·0075 % and the bile 0·02 %.

The merest trace of manganese was found in the gall-bladder of G.E., while in foetus F the bladder gave a strong line (0·0015 %) and in the dried bile the metal was present in even larger amount (0·008 %). Faint lines of rubidium were found in all the specimens. The bile of foetus F showed traces of silver, this metal being also found in the liver.

Pancreas. From Table II it will be seen that copper was present in every specimen examined, but that in only one case, that of the full-term foetus, did it rise to 0·002 % of the dry weight. Manganese was found in all cases,

and also rubidium, the lines of which are visible in every spectrum of the pancreas, but nowhere represent a value of more than 0·0024 %. The rubidium lines are weakest in the foetal specimens. Traces of silver were found in three cases. The strength of the manganese lines is the most striking feature of the examination of this organ.

Table II. *Pancreas.*

Patient	Copper	Manganese	Rubidium	Silver
S.	Trace	0·0008	0·0016	Trace
F.M.	Trace	Trace	Trace	Trace
A.B.	Trace	0·001	0·0016	Trace
G.E.	0·002 (almost)	0·0008	0·0024	—
N.C.	Trace	0·0008 (almost)	0·0016	—
Foetus D	0·002	0·0008 (almost)	0·0016	—
Foetus F	0·002	0·001	0·002	—
Foetus D (*a*)	Trace	Trace	Trace	—
G.C.	Trace	0·0008	0·0016	—
R.M.	Trace	0·0008	0·0024	—

Table III. *Spleen.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	—	0·0016	—	—
L.	Trace	—	0·0016	—	—
F.M.	Trace	—	0·0016	—	—
K.T.	0·002	0·0008	0·001	—	Trace of lithium
A.B.	Trace	—	0·0024	—	—
G.E.	Trace	—	0·004	Trace	—
N.C.	Trace	Trace	0·0024	—	—
E.C.	Trace	—	0·004	—	—
Foetus D	Trace	Trace	0·005	—	—
Foetus F	0·002	—	0·004	—	—
Foetus D (*a*)	Trace	—	0·004	—	—
G.P.	Trace	Trace	0·006	—	—

Spleen. As was expected, the iron lines were very strong in the spectra of this organ. The magnesium lines showed nothing remarkable. Copper was present in every specimen, reaching a concentration of 0·002 % in K.T. (a girl age 2) and in the full-term foetus. Manganese was only found in 5 out of the 12 spleens examined. In the highest (K.T.) it only reached 0·0008 %, while in all the others it was present in the merest traces. Rubidium occurred in all specimens. In the transfused guinea-pig it reached the high value of 0·006 %, and it was nearly as high in the foetuses. In the blood rubidium was found in the red cells, and the destruction of these by the spleen may account for the richness of the spleen in this element. Silver occurred in one case only, and a trace of lithium was found in the spleen of K.T.

Kidney. In the guinea-pig and in foetus D the potassium lines were of the same intensity as in the organs already described, but in all the other specimens they were distinctly weaker. The magnesium line was strong in foetus D (0·2 %), but in all the other specimens represented 0·1 %. Traces of copper were present in every specimen, reaching the concentration of the weakest standard solution in four cases, two of which were foetal specimens. Manganese was absent from the kidneys of the two foetuses which died

Table IV. *Kidney.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	Trace	0·0024	—	—
L.	0·002	Trace	Trace (faint)	—	—
F.M.	Trace	Trace	0·0024	—	—
K.T.	Trace	Trace	0·0024	—	—
A.B.	Trace	0·0008	0·003	—	—
G.E.	Trace	Trace	0·004	Trace	Lead (trace)
E.C.	Trace	Trace	0·003	Trace	—
Foetus D	0·002	Trace (faint)	0·005	—	—
Foetus (10)	0·002	—	—	—	Strontium (trace)
Foetus F	Trace	Trace	0·003	Trace	—
Foetus D (*a*)	Trace	—	0·0024	—	—
G.C.	Trace	0·0008	0·0024	—	—
G.P.	0·002	0·0008	0·004	—	—

in utero, but was present in small amount in all the other kidneys. Rubidium was absent from the kidney of the 10-weeks foetus and almost so from L. (myeloid leukaemia), but was present in significant amount in the other specimens. Traces of silver were found in three of the kidneys and lead in one. The kidney of the 10-weeks foetus was remarkable for containing a trace of strontium.

Table V. *Suprarenals.*

Patient	Copper	Manganese	Rubidium	Silver
S.	0·002	0·002	Trace	Trace
F.M.	Trace	Trace (faint)	Trace	Trace
K.T.	Trace	Trace (faint)	Trace	Trace
E.C.	Trace	Trace	0·003	Trace
Foetus D	Trace	Trace	0·006	—
Foetus F	Trace	Trace	0·0016	—
Foetus D (*a*)	0·002	Trace	0·004	—
Foetus L (2)	Trace	Trace	0·0016	—
G.P.	0·002	0·001	0·004	—

Suprarenals. Copper occurred throughout in small amount. Manganese was found in every case, but the variations were extraordinary (from a trace to 0·002 %). Rubidium occurred in small quantities in the post-natal specimens, while in the foetal ones measurable quantities were present. Silver was absent from the guinea-pig and foetal suprarenals, but occurred in traces in the others.

Urinary bladder. This organ was examined in two cases (A.B., G.E.) A trace of copper was present in each. Manganese occurred in A.B. as an obvious trace, while in G.E. the manganese line was only just visible. Rubidium was found in both specimens.

Intestinal canal. The stomach of F.M. and the duodenum of A.B. were examined. Traces of copper, manganese, and rubidium were found in both. A gastric ulcer removed by partial gastrectomy, compared with the adjacent normal stomach, showed a difference only in rubidium (normal 0·006 %, ulcer 0·0024 %), and in copper, in which element the ulcer was slightly richer. The Mn line in both cases was below the brightness of 0·0008 %. It is hoped to pursue this line of investigation in other cases.

Prostate. (M.L.) This organ contained traces of copper and silver, with 0·002 % of rubidium, but otherwise showed no unusual features.

Testis and epididymis. (M.L.) Both these organs contained traces of copper and the faintest traces of manganese. Rubidium was found in each (0·0016 %).

Uterus. (N.C., F.M.) The two specimens examined revealed no unusual features. Both contained traces of copper. Manganese was present as a trace in the uterus of F.M. but not of N.C. Rubidium was present in N.C. in a concentration of 0·0024 %, while in F.M. only a trace was found.

Placenta. The healthy 24-weeks placenta belonging to foetus D and that of foetus L (2) (myeloid leukaemia in the mother) were examined. Traces of copper and rubidium were present, but there was no manganese.

Ovary. (F.M., N.C., M.R.) The K lines were weak in F.M. Copper was present in small amount in all three, but the lines were distinctly stronger in the two patients who were below the age of the menopause. Manganese was absent, except for the merest trace in one of the ovaries of M.R. Rubidium gave a faint line (0·0016 %) in F.M. and slightly stronger lines (0·0024 %) in the other two. While there is obviously insufficient material for a generalisation, it is to be noted that the ovaries whose functional activity had not ceased were distinctly richer in potassium, copper, and rubidium, and a re-examination of the spectra shows that the same is also true of magnesium. There was, however, no difference between the spectra of the ovaries of the girl of 7 and of the adult of 27. The nature of any such changes in the mineral content of the ovaries which may set in after the menopause is under investigation.

Thyroid. (S, M.R., M.L., N.C. In the last case both the normal gland and a small adenoma were examined.) Copper was found in every case, but only in M.R. did it rise to a value of 0·002 %. Manganese was present in minute quantities in S. and in both specimens from N.C. but was absent from the other two. The rubidium lines were remarkably constant in all five specimens (0·002 %). Traces of silver were found in all the specimens. The normal gland was slightly richer in copper and the adenoma in manganese, but the differences were very small, and apart from these the spectra were identical. The thyroid of M.R. showed phosphorus, the lines of which were not visible in the other spectra.

Thymus. (Foetus D and foetus F.) The Mg line was stronger in the full-term foetus and the Cu lines were very much stronger (0·002 % as against a trace). The younger foetus, on the other hand, contained a trace of manganese, which was not present in the thymus of the elder. The Rb lines were strong in both (0·006 % in foetus D and 0·004 % in foetus F).

Tonsil. Four tonsils removed from children were examined. The spectra revealed practically no individual differences, but their general nature is interesting. Traces of copper were present in all but no manganese was found. The Rb lines were strong (0·003 %) and present in all. Every tonsil contained traces of silver.

Skin. The adult skin has not been examined, but the skin of foetus D contained 0·0024 % of rubidium, with traces of copper and manganese.

Heart. In all cases, pieces of heart muscle from the left ventricle were examined.

Table VI. *Heart.*

Patient	Copper	Manganese	Rubidium	Silver
S.	0·002	Trace	0·004	—
F.M.	0·002	Trace	0·004	—
K.T.	Trace	—	0·003	—
A.B.	Trace	—	0·004	—
G.E.	Trace	Trace	0·003	Trace
N.C.	Trace	Trace	0·003	Trace
E.C.	0·002	—	0·003	—
Foetus D	Trace	—	0·006	—
Foetus F	Trace	Trace	0·004	—
G.C.	Trace	—	0·002	—
R.M.	Trace	Trace	0·003	—
L (2)	0·002	—	0·0016	—
G.P.	0·002	Trace	0·006	—

Copper occurred throughout, but in only four human specimens was it more than a trace. Manganese was found in traces in six cases. Rubidium, on the other hand, gave lines that were remarkably constant in strength, corresponding to an average content of 0·0037 %. Silver was found in two of the specimens. No significant differences could be detected in the spectrum of E.C., death in this case being due to toxic myocarditis following diphtheria.

Aorta. (S., F.M., N.C.) As compared with N.C. (age 27) the Ca lines of S. (age 77) and F.M. (age 69) were markedly stronger, and, as is found in practically all the cases where the Ca lines are strong, the Mg line was much weaker. These changes are presumably the result of age. The merest traces of copper and rubidium were found in all three specimens. Manganese was only found in the aorta of S., and, as has been already mentioned, it gave a remarkably strong line (0·002 %). This aorta contained traces of silver and lead. Apart from any significance which may attach to the peculiar richness of this aorta in manganese, the presence of silver and lead in addition was presumably connected with the advanced degree of atheromatous change with calcification that was found.

Calcareous nodules from the case of S. were examined. These were found in the pericardium and on the mitral valve. The nodule on the valve was very small (0·168 g. dry weight) and it is possible that its general poorness in metals may be due to its small size making the traces present difficult of detection. The other nodule showed strong Cu lines (0·002 %), a very strong Mn line (0·002 %), and traces of silver and lead. It is evident from the changes found here and in the aorta that the process of calcification provides a nidus for the deposition of the heavy metals. Inasmuch as copper and manganese were present in such large amounts, one would have expected iron to be well represented, but this was not the case. The iron spectrum was absent from the smaller specimen, and only showed faint indications in the larger one.

Neither specimen contained rubidium. The K lines were distinctly weak. The Ca lines were naturally very strong, and the antagonism that appears to exist between calcium and magnesium was also found here, the Mg lines being very weak, indicating the presence of less than 0·02 % of this element.

Table VII. *Lungs.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	Trace	0·0024	Trace	—
F.M.	Trace	Trace	0·0024	Trace	—
K.T.	Trace	—	0·0016	Trace	—
A.B.	Trace	Trace	0·0024	Trace	—
G.E.	Trace	—	0·0016	—	—
N.C.	Trace	Trace	0·0016	—	Lead (trace)
E.B.	0·002	Trace	0·0016	Trace	—
Foetus D	Trace	—	0·006	—	—
Foetus (10)	0·002	—	—	—	Strontium (trace)
P.	Trace	—	0·0024	—	—
Foetus F	0·002	Trace	0·0024	—	—
G.P.	Trace	Trace	0·006	—	—

Lungs. A noteworthy feature of the lung spectra is the strength of the iron spectrum. The difficulty of washing this tissue free of blood does not account for the feature entirely, as in the transfused guinea-pig the iron lines of the lung were stronger than those of any of the other organs with the exception of the spleen. In E.B. and in P. (both of which were cases of silicosis, E.B. being a very advanced one) the iron spectrum was very rich, in the case of E.B. being exceptionally so. Indeed, in E.B. the Fe lines were stronger than those obtained by burning the same weight of dried normal blood-clot, indicating that the silicosis was accompanied by a definite siderosis. The Mg line was faint in both these specimens. Copper was present in all the specimens, but only in E.B. and the two foetuses did it rise to 0·002 %. Manganese, in traces only, was found in 7 of the 12 lungs. Rubidium was found in 11 specimens without great variation in quantity except in foetus D and the transfused guinea-pig, both of which showed high values. Faint traces of silver were found in five specimens, and traces of lead and strontium each in one case.

Table VIII. *Muscle.*

Patient	Copper	Manganese	Rubidium	Silver
M.R.	Trace	—	0·006	Trace
M.L.	Trace	—	0·006	Trace
Foetus F (pectoral)	0·002	Trace	0·0024	—
Foetus F (diaphragm)	Trace	Trace	0·003	—
Foetus D (quadriceps)	Trace (faint)	—	0·004	—
G.C.	Trace	—	0·006	—
R.M.	Trace	—	0·006	—

Muscle. The Mg lines are weaker in the foetal specimens (0·1 %) than in the adult (0·2 %). Copper was found in all cases in small amounts, but the Cu content of the full-term foetus shows one feature which may be of importance. Pieces were taken from the pectoralis major and the diaphragm, and there is a distinct difference in the copper contents of the two, that of the diaphragm being the lower. The foetus was stillborn and had never

breathed, which suggests that the greater amount of copper in the pectoral muscle may be connected with its functional activity during the movements of intra-uterine life. In the adult muscles copper occurs in traces only, but all the organs are alike in showing a greater content of copper in the foetus than in the adult. Manganese was only found in the specimens from the full-term foetus. Rubidium occurred throughout, reaching a constant degree of concentration which is only equalled by the heart muscle and by the liver. Traces of silver were found in two of the specimens.

Calculi. Two calculi were examined, a single large cholesterol stone from the gall-bladder, and a small phosphatic calculus from the kidney.

The cholesterol stone contained the merest trace of magnesium, potassium, calcium, and iron, but a notable amount of copper was present (0·035 %). This figure agrees well with those found by Schonheimer and Oshima [1929] in their chemical analysis of six gall-stones, which varied between 0·016 % and 0·2 % Cu.

In the renal calculi the Ca lines were very strong, while both the K and Mg lines were weak. Manganese and rubidium were absent, but traces of silver and lead were found. There was only a faint indication of copper. The most remarkable feature is the presence of a well-marked Li line (0·0003 %). It is to be noted that the spectra of this and of the spleen of K.T. alone out of all those examined show a Li line. Some investigations now in progress, however, appear to show that this element is frequently found in the urine.

Cartilage. Five specimens of cartilage were examined. (Trachea from F.M., G.E.; rib cartilages from K.T., foetus D and foetus F.)

In G.E.—a man of 53—the K and Mg lines were weaker than in the others, while the Ca lines were stronger, presumably indicating a senile calcification. Copper was found in traces only in all the specimens except the full-term foetus (0·015 %). A trace of manganese was found in K.T. only. Rubidium occurred in all the specimens, the amount being smallest in G.E. while in the others it gave lines of approximately equal strength representing 0·003 %. A trace of lead was found in G.E. and the merest traces of iron were present in all five spectra.

Bone. (Parietal bone from K.T. and foetus D.) In both instances the weakness of the K and Mg lines and the strength of the Ca lines were the most prominent features of the spectra. Copper was just detectable, but manganese and rubidium were absent. The bone from K.T. contained a trace of lead.

Pituitary. (S.) Copper and manganese were both present in amounts of less than 0·002 % and 0·0008 % respectively. An infinitesimal trace of rubidium was found.

Brain. (Cerebral hemispheres S., K.T., foetus D., cerebellum K.T.)

The Mg lines were prominent throughout, and especially so in the case of the foetal brain (0·25 %). The Mg lines were stronger in the brain than in any other foetal organ. Copper was found in all the specimens, being strongest

in the foetal brain (0·0035 %). Manganese was absent from all, save for a very faint trace in the cerebrum of K.T. Rubidium was found in all cases. Traces only were present in the brain of K.T. but in the cases of S. and foetus D this element reached values of 0·004 and 0·003 respectively. Comparing the cerebrum with the cerebellum of K.T., the former, in addition to containing a trace of manganese, was definitely richer in both copper and magnesium.

Teeth. Seven teeth were examined, one of which was an incisor of the first dentition. In the adult teeth, no differences were found between the incisors, molars, and bicuspids. The spectra showed the following features.

(*a*) A very low magnesium content, this line being only just visible. As would be expected the Ca lines were very strong, and were stronger in the crown of the tooth than in the fang.

(*b*) The teeth were rich in sodium, but were low in potassium. The K lines indicated a content of less than 0·08 %, as against about 0·6 % for an organ like the liver.

(*c*) The iron spectrum was so faint as to be almost invisible, but the lines due to copper were quite clear. It will have been noticed that all the foetal organs and tissues appear to be much richer in copper than the adult ones, and this difference applies equally to the teeth. In the deciduous tooth, the copper amounted almost to 0·002 %, while in the adult teeth, the lines, though clearly visible, were much weaker. Manganese was not found.

Milk. Five specimens of human milk were taken, but unfortunately the stage of lactation they represented was not noted. There was, however, no specimen of colostrum. All the milks showed lines due to sodium, potassium, magnesium, and calcium. There was no marked variation in these metals except that three of the milks were lower in both potassium and magnesium than the other two. Iron was found in minute traces in all. Copper was present in all the specimens, in amounts of less than 0·002 % of the dry weight, except in one instance, where it just reached this value. Traces of rubidium occurred in two out of the five specimens (0·001 %), but manganese was not found in any.

Carcinoma. Only one specimen of carcinoma has been examined in this series—a squamous-celled carcinoma of the oesophagus. Traces of copper and rubidium were found, but no manganese. The spectrum apart from these features, showed nothing unusual.

Blood. The results of the examination of the blood will be found in Table IX. In the case of blood and serum the examination was carried out on 0·1 cc., and in the case of the solid clot, on 0·05 g. of the dried material. No attempt has yet been made to examine the plasma as such, as it was feared that by the use of either vaselined tubes or citrate to prevent clotting, impurities might be introduced. The method adopted was to allow the blood to clot and the serum to separate. This was removed by pipette and centrifuged. The clot was then washed rapidly in distilled water and placed in the hot steam-oven.

In a few minutes a pool of serum was expressed from the clot. This was removed, the clot broken up and again rapidly washed in distilled water, before being finally dried. The method does not, of course, effect a complete separation of the clot from the serum, but it was thought best at this stage to employ it, rather than introduce the use of chemicals.

Table IX. *Blood.*

Disease	Age	Sex	Copper	Manganese	Rubidium	Silver
1. Anaemia of pregnancy	40	F.	0·002	—	—	Trace
2. 37 weeks pregnancy	—	F.	Trace	—	Trace	—
3. Full-term ,,	—	F.	Trace	Trace	Trace	—
4. 36 weeks ,,	—	F.	Trace	—	Trace	Trace
5. 38 weeks ,,	—	F.	Trace	Trace	Trace	—
6. 12 weeks ,,	—	F.	Trace	Trace	Trace	—
7. 22 weeks ,,	—	F.	Trace	Trace	Trace	Trace
8. Diffuse hyperplastic sclerosis	40	F.	Trace	—	—	—
9. Myeloid leukaemia	?	F.	Trace	—	—	—
10. Myeloid leukaemia. Clot	30	M.	Trace	—	0·006	—
11. ,, Serum			Trace +	Trace	—	—
12. Foetus D. Heart blood (Dried whole blood)	—	—	Trace	—	0·004	—
13. Foetus F. ,, (Dried whole blood)	—	—	0·005	—	0·004	—
14. Foetus D (*a*). Liver blood (Dried whole blood)	—	—	0·005	—	0·002	—
15. 22 weeks pregnancy (clot)	—	F.	Trace	—	0·003	Trace
16. ,, (serum)	—	F.	Trace	Trace	—	—
17. Paget's disease of bone (0·1 cc. blood)	25	M.	Trace	Trace	0·0016	—
18. ,, (serum)			Trace +	Trace	—	—
19. ,, (clot)			Trace	—	0·003	—
20. Myxoedema	35	M.	Trace	—	Trace	Trace
21. Carcinoma of ovary	28	F.	0·002	Trace	Trace	—
22. Chlorosis	15	F.	0·002	Trace	Trace	—
23. Tabes dorsalis	38	F.	Trace (faint)	Trace	—	—
24. Cirrhosis of liver	63	F.	Trace (faint)	—	—	—
25. Perinephric abscess	35	M.	Trace (faint)	—	—	—
26. Pernicious anaemia	60	M.	Trace (faint)	Trace	—	—
27. Polycystic kidney	36	M.	Trace (faint)	Trace	—	—
28. Landry's paralysis	24	M.	Trace (faint)	Trace	—	—

The iron spectrum was strong in all the specimens of blood, with individual variations depending on the presence of anaemia. A very faint trace of iron was also seen in the centrifuged serum. Sodium appeared to be present in the serum in greater amount than in the clot. Potassium, on the other hand, was found chiefly in the red cells, the potassium spectrum of the serum being very weak. Copper occurred in all the specimens. It reached a strength that could be compared with the weakest standard solution in two of the three foetal cases, but only one of the adult bloods was sufficiently rich in copper to do this. It happened that this—a case of severe anaemia of pregnancy—was the first blood to be spectrographed, and the finding of an unusual amount of copper led to six other specimens of blood from cases of normal pregnancy being examined. The fact that the foetal tissues are so rich in copper, which must be supplied by the mother, gave rise to the expectation that pregnant blood might, in general, be characterised by such a richness in copper as was found in this case. The copper contents of the six pregnant bloods were higher than in the other adult bloods, but nowhere as high as in the anaemia of pregnancy. Evidently the large demands made by the foetus for copper cause

this slight increase in the level of copper in the blood during pregnancy. The association in one case of a severe anaemia with an unusually high level of copper suggests that for some unknown reason there was an unusual mobilisation of the copper reserves of the mother, and our present knowledge of the relation between copper and the formation of haemoglobin suggests that it was the depletion of this which led to the anaemia.

In all the specimens examined, the copper appeared to be equally distributed through the serum and the clot. Manganese was found in eight of the specimens. In the three cases where clot and serum from the same blood were examined, the manganese was invariably found in the serum, but not in the clot. Rubidium, on the other hand, showed the reverse distribution, being found in the clot but not in the serum.

Traces of silver were found in the blood of five patients. It only happened to occur once in the cases where both clot and serum were examined, and then it was found in the clot, but not in the serum.

The bloods from myeloid leukaemia, myxoedema and diffuse hyperplastic sclerosis were not distinguished by any peculiar features. Eighteen sera which had been tested for the Wassermann reaction were examined, but no differences were noted between the positive and negative sera.

Lymph-gland. A gland from L. (lymphatic leukaemia) showed traces of copper and rubidium, but no other features of note. A partially calcified tuberculous gland from the mesentery of another patient, showed a trace of manganese in addition.

Bone-marrow. Marrow from the middle of the shaft of the femur of a man of 25, who died of miliary tuberculosis, was examined. The spectrum is poor in potassium, and neither manganese nor rubidium is present. Copper amounted to slightly less than 0·002 %.

Discussion.

The detailed description of the various human tissues shows that in addition to sodium, potassium, calcium, magnesium and iron, copper is a universal constituent of protoplasm, rubidium is almost universal, while manganese and silver, in addition to an apparently irregular distribution, are also constant constituents of certain organs. Each of these metals will be discussed separately.

Copper.

Every specimen taken from living tissue contains copper, though in a tissue such as bone the amount present is very small. The presence of copper in the tissues of plants and animals has been recognised for a long time, its occurrence being regarded as of either accidental or toxic nature, and it is only in recent years that the possibility of its being an element essential to life has had to be considered. The well-known work of Waddell *et al.* [1928, 1929], demonstrating the essential rôle played by copper in the formation of

haemoglobin, is in keeping with the trend of other recent work which has demonstrated the widespread distribution of copper in living tissues [Lindow, Elvehjem and Peterson, 1929]. In a recent paper, Fox and Ramage [1931] found copper to be present in every one of 146 specimens of tissues removed from different species of invertebrates. In the present investigation, copper has been found in every one of 195 specimens of tissue removed from 23 human beings. The constant presence of copper in over 500 tissues from all parts of the animal and vegetable kingdoms, together with the evidence of McHargue [1925] and Maquenne and Demoussy [1920] as to its distribution in the plant world, leads to the conclusion that copper is a universal constituent of protoplasm. That it is also an essential constituent is rendered likely by its known importance in relation to haemoglobin formation in mammals and its occurrence in haemocyanin.

There are certain aspects of the distribution of copper in human tissues which merit separate attention.

(1) It is obvious that copper is of great importance in the functions of the liver during foetal life. The copper values in the five foetuses examined are between seven and ten times in excess of the adult percentage. This is in agreement with the finding of McHargue [1925] who in the liver of an ox found 50 mg. of copper per kg. of dry tissue, while in that of a calf born dead the figure was 908 mg. of copper per kg. It is probable that part at any rate of the peculiar importance of copper for the foetal liver may be connected with its blood-forming functions in this stage of life.

(2) The other foetal organs also contain a greater amount of copper than the corresponding adult tissues, though the difference is far less than in the case of the liver. This has been found by other workers. Thus Bodansky [1921] found that the copper content of the foetal brain is greater than that of the adult. Copper presumably plays an important part in the phenomena of growth. This seems to be the most feasible explanation of the difference in the copper contents of an incisor of the first dentition and of various teeth taken from adults. In view of the knowledge that is being accumulated by the work of Mellanby [1931] and others on the relation between an adequate vitamin supply and the formation of sound teeth, and the suggestion of McHargue [1925] that copper may play some part in the formation of certain of the vitamins, the relations that may exist between copper and the growth of the teeth merit further investigation.

(3) It is obvious that to supply the great demands of the foetus for copper, inroads must be made on the maternal store of this metal, unless a sufficient amount to meet the foetal demands is present in the food during pregnancy. The drain of copper from the mother does not cease after birth, for the examination of five specimens of human milk showed copper to be present in all. That it would be possible to take a diet deficient in copper is indicated by the investigation of Lindow, Elvehjem and Peterson [1929]. It therefore seems possible that the chronic secondary anaemia and general debility found

in some cases of pregnancy may be connected with a dietary deficiency of copper. The whole question of the metabolism of copper during pregnancy seems a promising field for investigation. Its probable importance is further suggested by the solitary observation that, of the pregnant bloods that were examined, the greatest amount of copper occurred in a case of severe secondary anaemia, which may have been due to some unusual demand of the foetus for copper leading to an abnormal mobilisation of the maternal store of this metal.

(4) Of the various diseases that were investigated, the only ones associated with marked abnormalities of copper content were those represented by R.M. (chronic interstitial nephritis) and J. (atrophic cirrhosis of the liver). In the absence of more extended knowledge, the meaning of the large amount (0·02 %) of copper in the livers of these cases must be left an open question.

(5) The presence of copper in bile (and in a gall-stone) suggests that this is a channel of excretion for the metal. In the case of two sheep that were examined, the copper lines were visible on burning 0·1 cc. of bile, while the average content of their gall-bladders (dry) was 0·005 %. The high figures also obtained in the human gall-bladders suggest that the body attempts to conserve its supply of copper by reabsorption through the wall of the gall-bladder, and this probably occurs through the intestine as well.

Manganese.

(1) Manganese is especially found in the liver, pancreas, kidney and suprarenal, and to a less extent in the heart and lung. Its distribution through the other tissues is irregular. This differs in several respects from the findings of Zbinden [1930], who obtained manganese only in the liver and kidney. Our results agree in showing that the liver contains the greatest amount of this metal.

(2) The high content in manganese of the tissues from the transfused guinea-pig is noteworthy.

(3) Of the human specimens, the greatest individual amounts are found in the tissues of S. All the organs contain normal amounts with the exception of a calcified nodule from the heart, the suprarenals, and the aorta. This man died from cerebral haemorrhage at the age of 77, and the unusually high amounts of manganese in the aorta and suprarenals especially, raises the interesting possibility that the development of arteriosclerosis may be connected with the metabolism of manganese.

(4) The examination of the liver of J. lends no support to the thesis that cirrhosis of the liver may be due to chronic manganese poisoning.

(5) Manganese was found in 14 out of 28 specimens of blood, its presence being confined to the serum.

Rubidium.

Rubidium lines were found in 16 of the 28 bloods examined. Rubidium is present in the cells, and not in the serum.

(1) Rubidium has a very much wider distribution than manganese in the tissues. This may be due to its greater diffusibility.

(2) The distribution of rubidium is, in general, the opposite of that of manganese. While the latter is chiefly found in the glandular organs such as the liver and pancreas, being present in traces in 7 only of the 12 hearts and in 1 only of the 6 specimens of muscle, rubidium reaches its greatest concentrations in the heart and striated muscle.

(3) The only tissue from which rubidium is absent in the adult is bone.

(4) The only individual to be without rubidium was the 10-weeks foetus. It is impossible to say whether this is a chance result, or due to the early age of the foetus, or to be associated with other mineral abnormalities that were present.

(5) In general, this element appears to be associated with potassium in its distribution through the tissues, the strength of the lines due to the two metals tending to vary harmoniously. It must be borne in mind, however, that the brightness of the lines of these two elements may not be entirely independent quantities, since Gooch and Phinney [1892] by eye-observations on low temperature flames have shown that the presence of potassium salts in minute quantities will materially increase the brightness of the rubidium spectrum, while the reverse occurs when the potassium is present in larger amount.

(6) Rubidium has been found in this investigation to be almost universally present in tissues. The fact that Zbinden [1930] makes no reference to it in the course of his work is probably due to the difference in technique—since he uses an arc spectrum while we employ a flame spectrum—and in a paper on milk he states that rubidium "ne donne pas de raies spectrales assez sensibles entre 2500 and 3500 Å."

The trend of previous work on the physiological behaviour of rubidium is to show that the metal can partially or completely replace potassium in Ringer's solution. Ringer himself [1883] noted this action, and it has been confirmed by Zwaardemaker [1919] and others. Loeb [1920] finds that rubidium chloride is able entirely to replace potassium chloride in the solution required for the development of the eggs of the sea-urchin into swimming blastulae. Mitchell *et al.* [1921] by perfusing frogs with Ringer's solution in which the potassium chloride was replaced by rubidium chloride found that the rubidium was taken up by muscles which had been kept in activity, but was not taken up by resting muscle. In feeding rats with rubidium, they found that the bulk of this metal was stored in the muscles, which is in agreement with the findings of the present paper. Mendel and Closson [1906], after the injection of rubidium salts into rabbits and cats, came to the same conclusion, stating that "muscles form the chief storehouse for the temporary retention of rubidium, in which they surpass the liver." That the inter-availability of potassium and rubidium in this way is not the whole story is shown by further experiments of Mitchell and Wilson who fed young white

rats on a purified synthetic diet in which the salts of potassium were replaced by those of rubidium. After 10 to 15 days the rats showed marked derangement, with irritability and tremblings, developing later into tetanic spasms, and death within 48 hours. In all these experiments it was also found that caesium behaved like rubidium in all respects. The physiological behaviour of both caesium and rubidium so far as it is known, appears to be identical, yet caesium has not been found in any of the present series of tissues, or in a series of examinations made by Fox and Ramage [1931] on animals of widely different genera. It is difficult to say whether this is due to some unknown difference between the physiological properties of the two elements or to the fact that caesium, though as widely distributed in nature as rubidium, is not present in such great amounts. In sea-water the proportion of caesium to rubidium is about 1:200. It is possible that it may be present in living tissues, but in such small amounts that very much larger quantities would have to be burnt than were employed here, in order to demonstrate its presence. The presence of rubidium in tissues has a further interest in that it also occurs in sea-water, the relationships between the mineral composition of blood-plasma and sea-water being a well-known phenomenon. Rubidium may prove to be of further importance in that it is a faintly radio-active element. In this respect it resembles potassium. Zwaardemaker [1919] finds that rubidium can replace potassium in Ringer's solution in proportion to their relative radio-active powers. Roffo and Landaburu [1925] find that the radio-activity of organs injected with rubidium chloride is variable, but that tumour tissue always shows a higher radio-activity than normal tissue, presumably owing to the fact that tumour tissue absorbs more rubidium.

Silver.

Silver is present in considerably less quantity than the preceding metals, never rising to 0·001 % of the dry weight. It was found in every specimen of thyroid and tonsil examined (8 in all). It was found in the other organs in the following percentage of cases: liver, 47 %; suprarenal, 44 %; lung, 36 %; muscle, 33 %; pancreas, 30 %; kidney, 23 %; heart, 14 %; spleen, 8 %; isolated instances occurred in the aorta and a calcareous nodule. It was absent from the rest of the tissues. In blood, traces of silver were found in 5 out of 28 cases. Its presence does not appear to be connected with age and is not universal, since it is absent from all the tissues examined from foetus D and from the case of R.M., though in neither of these were either the tonsils or thyroid examined. None of the guinea-pig specimens contained silver. Whether this element has any physiological function, or whether its presence is accidental, its irregular distribution being due to selective absorption by the different tissues, is uncertain. Zbinden [1930] using the arc spectrum has found silver in every human tissue he has examined, including blood; thyroid being especially rich in silver, with which these investigations are in agreement.

Lead.

This occurred only spasmodically. It was found in the liver, kidney and cartilage of G.E., the aorta and a calcareous nodule of S., the bone of K.T., the renal calculus and in the lung of N.C. This varied and irregular distribution strongly suggests that the metal is present accidentally.

Strontium.

This metal was found in the kidney of the guinea-pig, and in the liver and lung of the 10-weeks foetus. This scanty amount does not suggest that the metal has any physiological functions, and it has not been described by Zbinden [1930] as being found by his spectroscopic method. Some unpublished observations of ours, however, indicate that strontium is probably a constant constituent of the retina. It is curious that in the present investigation the 10-weeks foetus should provide two out of the three instances where this element occurs. The whole mineral content of this foetus appeared to be abnormal, being deficient in potassium, devoid of rubidium, and containing strontium and a high proportion of calcium. It would be interesting to enquire whether such early foetal death may, in other cases, be associated with abnormalities of their mineral constitution, or if the composition found is normal for that age.

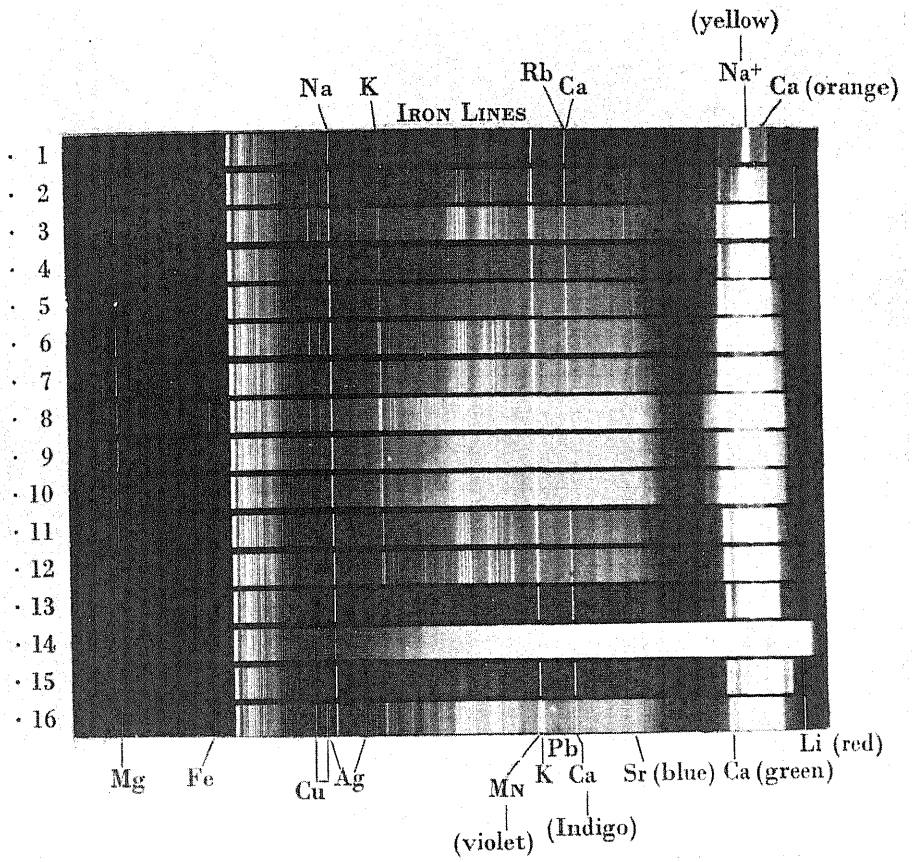
Lithium.

In view of its wide distribution through the animal kingdom, it was expected that this element would be of frequent occurrence, and its rarity came as a surprise. A lithium line was only found in two instances—a renal calculus and the spleen of K.T. Further work is required, since Hermann [quoted by Zbinden, 1930] states that it occurs in the foetus, and in the adult is concentrated particularly in the lungs, while Keilholz [1921] actually gives a table of values for the lithium content of the various organs. Zbinden [1930], however, makes no reference to lithium. In the course of some further work, we have found lithium to occur in human urine. It is possible that the differences which have been found are related to local variations in the composition of the soil or the water supply.

SUMMARY.

1. Copper is a universal, and is probably an essential, constituent of living tissue. It occurs in greater concentration in foetal tissues than in adult, and reaches its maximum amount in the foetal liver, where the percentage of copper may be as much as ten times in excess of that present in the maternal liver.

2. Manganese occurs spasmodically in many tissues, but is chiefly concentrated in the liver, pancreas, suprarenal and kidney. It is present in bile, foetal bile containing a high proportion. In the blood it appears to be confined to the serum.



No. 1. 0·02 cc. standard solution.
2. 0·05 cc. standard solution.
3. 0·1 cc. standard solution.
4. Pancreas. Foetus D.
5. Gall-bladder and bile. Foetus D.
6. Liver. Foetus D.
7. Suprarenal. Foetus D.
8. Blood from liver. Foetus D.

No. 9. Kidney. Foetus D.
10. Spleen. Foetus D.
11. Quadriceps muscle. Foetus D.
12. Blood (clot). Myeloid leukaemia.
13. Blood (serum). Myeloid leukaemia.
14. Gall-stone.
15. Renal calculus.
16. 0·2 cc. standard solution.

3. Rubidium is almost as widely distributed as copper and occurs in greatest concentration in the heart and in striated muscle.

4. Silver is found to a less extent than the preceding metals, but appears to be a normal constituent of the thyroid and the tonsil.

5. Lead, strontium and lithium were only found to occur spasmodically.

It is hoped to pursue the various points that have been raised in the course of this investigation.

We wish to express our thanks to all those who have helped in the supply of tissues—to Dr S. C. Dyke, Director of the Pathological Department, both for the supply of material and for allowing the use of the Laboratory for part of the work, to Dr W. Stewart, the assistant Pathologist, to Mr Maslen Jones and Dr Wilkes of the Maternity Department for their help in obtaining foetal tissues and to the Royal Society Government Grant Committee for grants for the purchase of spectrographic apparatus.

REFERENCES.

Bodansky (1921). *J. Biol. Chem.* **48**, 361.
Fox and Ramage (1931). *Proc. Roy. Soc. Lond.* B **108**, 157.
Gooch and Phinney (1892). *Amer. J. Sci.* **43**, 392.
Hermann (quoted by Zbinden, 1930. *Pflüger's Ber.* **109**, 26.)
Keilholz (1921). *Pharm. Weekblad*, **58**, 1482.
Lindow, Elvehjem and Peterson (1929). *J. Biol. Chem.* **82**, 465.
Loeb (1920). *J. Gen. Physiol.* **3**, 229.
McHargue (1925). *Amer. J. Physiol.* **72**, 583.
Maquenne and Demoussy (1920). *Compt. Rend. Acad. Sci.* **170**, 87.
Mellanby (1931). *Brit. Med. J.* i, 507.
Mendel and Closson (1906). *Amer. J. Physiol.* **16**, 152.
Mitchell, Wilson and Stanton (1921). *J. Gen. Physiol.* **4**, 141.
Ramage (1929). *Nature*, **123**, 601.
Ringer (1883). *J. Physiol.* **4**, 370.
Roffo and Landaburu (1925). *Physiol. Abstr.* **10**, 305.
Schonheimer and Oshima (1929). *Z. physiol. Chem.* **180**, 249.
Waddell, Steenbock, Elvehjem and Hart (1928). *J. Biol. Chem.* **77**, 777.
—— —— —— —— (1929). *J. Biol. Chem.* **83**, 251.
Zbinden (1930). *Mém. Soc. Vaud. Sci. Nat.* **3**, 233.
Zwaardemaker (1919). *J. Physiol.* **53**, 273.

CLXXIV. BLOOD-PHOSPHORUS IN FAT ABSORPTION.

By BO VAHLQUIST.

From the Institute of Medical Chemistry, University of Upsala, Sweden.

(*Received August 13th, 1931.*)

The view held that the phosphatides are concerned in the transport of fat for utilisation or deposition in the tissues seems to have found rather a general acceptance. To a great extent this view is experimentally based on the observation that the phosphatide content of blood is very often increased on occasions when an increased fat transport with the blood is presumably taking place, *e.g.* after fat ingestion. This phenomenon has been studied particularly by Bloor [1915, 1916], but has also been observed by many other investigators, usually in experiments on dogs[1].

According to Bloor [1916] most of the increase of the phosphatides takes place in the blood-corpuscles, in which this author found at the same time an augmented content of glycerides. On the strength of these observations Bloor advanced the interesting and much cited hypothesis that the fatty acids absorbed from the intestine are converted into phosphatides in the corpuscles and mainly as such are removed from the blood. Noticeably little additional work has, however, been done in order to test the correctness of this hypothesis. Knudson [1917] confirmed the observation of phosphatide increment in the corpuscles after fat ingestion. On the other hand Bloor [1920] himself in a later paper mentions that "further unreported work in this laboratory has not, however, entirely borne out these findings, since certain dogs have been found, in which the increase of lipoid phosphorus took place in the plasma and not in the corpuscles." Further Iwatsuru [1926] found no such increase (in experiments with rabbits). Under these conditions I have thought it desirable to submit the question of the alimentary phosphatide increase in the corpuscles and in plasma to renewed investigation. For a further trial of the hypothesis in question I have, concurrently with the determination of lipoid P, also determined the non-lipoid P of plasma and corpuscles. This was done with the idea that if the phosphatides added to the blood during fat absorption are formed in the blood itself, either in corpuscles or in plasma,

[1] It is noteworthy that no alimentary increase of blood phosphatides has been found in man [Hiller, Linder, Lundsgaard and Van Slyke, 1924; McClure and Huntzinger, 1928; Page, Pasternack and Burt, 1930]. This discrepancy is not unlikely to be explained by the fact that the human subjects tested in this respect have received, in spite of the ingestion of about 100 g. of fat, a much smaller quantity of fat in relation to their body weight than the dogs.

it might reasonably be expected that the non-lipoid P of the blood should show a simultaneous decrease.

Methods.

The investigations were performed on dogs which were starved for 24 hours before the test-meal. Between the experiments the dogs were fed on a mixed diet, for the main part consisting of meat. Blood (about 15 cc. each time) was drawn from a cutaneous vein on the hind leg just before the fat ingestion and 2, 4, 8, 12 and generally also 24 hours afterwards. The test meal consisted of olive oil in an amount of about 5 cc. per kg. body weight (usually 100–120 cc. in all) and of 100–200 g. of lean meat. The oil was given by stomach tube or simply by pouring it directly into the pouch produced in the mouth of the dog, by pulling the lips away from the jaws on one side. In Exps. 5, 6 and 7 the animals were given 100 g. of glucose in water (+ 0·25 g. of tannic acid) 18 and 12 hours before the fat meal (*i.e.* 200 g. of glucose in all). In Exp. 6 no meat was given. The blood was transferred to a flask containing solid sodium oxalate in a quantity of about 0·2 % of the blood taken. After the oxalate had been mixed up with the blood, this was centrifuged for 30 minutes at a rate of 2700 r.p.m. and the plasma cautiously drawn off. The leucocyte layer and the uppermost layer of the red corpuscles were removed and the rest of the erythrocytes taken for analysis.

For the phosphatide determination 2 cc. of plasma were dropped slowly into 40 cc. of a mixture of 3 parts of alcohol and 1 part of ether which was kept rapidly rotating. The mixture was heated to boiling in a water-bath and thereafter cooled to room temperature. The entire fluid was then filtered into a 100 cc. Erlenmeyer flask, two washings with 10 cc. of the alcohol-ether mixture being employed. The filtrates were evaporated to dryness on a water-bath, the residue incinerated with sulphuric and nitric acids and the phosphorus determined titrimetrically according to the modified Neumann method, described by Widmark and Vahlquist [1931].

To a weighed quantity (about 1·5 g.) of corpuscles was added an approximately equal amount of water and the mixture again weighed. The fluid was then shaken vigorously and allowed to stand for a few minutes. The haemolysed corpuscles were poured drop by drop into the alcohol-ether mixture and the amount poured in was determined by weighing before and after. For the rest the determination was performed in the same way as with the plasma.

In plasma the so-called acid-soluble P is practically identical with the non-lipoid-P. The acid-soluble P was determined as follows. 4 cc. (in some cases only 2 cc.) of plasma were added drop by drop to an Erlenmeyer flask containing ten times the amount of saturated picric acid in 0·25 % hydrochloric acid. After standing for 1 hour the mixture was filtered and an aliquot part (usually 30 cc. out of 44 cc.) was evaporated, incinerated and the phosphorus determined according to the Widmark-Vahlquist method.

In the corpuscles the non-lipoid P was determined as the difference

between total and lipoid P. For the determination of the total P of the corpuscles quantities of about 0·75 g. were weighed, thereafter incinerated and analysed for P as above.

The experimental confirmation of the accuracy of the methods used for the determination of lipoid P and acid-soluble P of plasma was given by Widmark and Vahlquist [1931]. For the quantities of plasma used in the present work the error does not as a rule exceed ± 0·05 mg./100 cc. With regard to the determination in the corpuscles the accuracy becomes somewhat less. According to our experience the error of the determination of lipoid P and of total P in the corpuscles seldom exceeds ± 0·2 mg./100 cc. and ± 1 mg./100 cc. respectively.

Table I. *Experiments with fat ingestion.*

		Plasma		Corpuscles		
Exp. No. Date Exp. animal	Hours after fat ingestion	Phos-phatide P mg./100 cc.	Acid-soluble P mg./100 cc.	Phos-phatide P mg./100 cc.	Non-lipoid P mg./100 cc.	Turbidity of plasma
I	0	—	—	14·2	48·6	0
20. viii. 1930	2	16·1	5·3	14·3	51·2	+ +
Dog 1. ♂	4	17·4	5·1	14·9	49·2	+ + +
	8	16·8	5·1	14·2	49·9	+(+)
	12	16·9	4·6	14·1	50·0	+
	24	14·2	3·1	14·3	47·1	0
II	0	15·3	3·9	16·6	53·6	0
27. viii. 1930	2	16·1	4·7	16·2	54·1	+ +(+)
Dog 1. ♂	4	17·4	5·4	17·0	50·8	+ + +
	8	16·6	4·7	16·8	51·8	+ +
	12	15·6	5·4	16·3	54·1	+
	24	14·5	3·3	16·5	51·7	0
III	0	14·7	4·5	16·7	48·8	0
3. x. 1930	2	14·4	5·0	16·5	51·0	(+)
Dog 2. ♂	4	16·9	6·2	16·0	48·0	+
	8	15·8	6·1	17·0	48·3	+(+)
	12	15·0	5·1	16·4	50·1	0
IV	0	12·3	3·3	14·6	—	0
26. x. 1930	2	12·0	3·3	13·8	—	+ +
Dog 3. ♀	4	14·4	4·0	15·0	—	+ + +
	8	13·3	3·3	14·5	—	+
	12	11·9	4·1	14·4	—	(+)
	24	11·6	3·7	14·2	—	0
V	0	12·2	3·6	15·5	51·0	0
12. x. 1930	2	12·8	3·4	15·2	49·0	+ +
Dog 1. ♂	4	14·4	3·8	15·3	50·8	+ + +
	8	15·4	3·8	14·6	49·4	+ + +
	12	15·5	4·4	14·7	52·7	+ +
	24	14·8	3·4	14·7	52·2	(+)
VI	0	9·7	3·7	14·4	—	0
17. xii. 1930	2	10·0	4·0	14·3	—	+(+)
Dog 2. ♂	4	11·1	—	14·5	—	+(+)
	8	11·4	3·7	14·1	—	(+)
	12	11·2	4·6	14·4	—	0
VII	0	9·7	4·3	14·7	—	0
20. xii. 1930	2	9·8	4·9	—	—	+
Dog 2. ♂	4	11·5	4·6	14·2	—	+ +(+)
	8	10·3	4·7	14·5	—	(+)
	12	9·9	4·9	14·4	—	0
	24	9·9	4·4	14·0	—	0

+ cloudy, + + slightly milky, + + + milky

Experimental results.

The analytical data are comprised in Tables I and II. In all experiments with fat ingestion (Table I) there is a clear increase of the plasma phosphatides, varying between 14 and 26 % of the fasting values. In five of the experiments the maximum is reached after 4 hours, in one experiment after 8 hours, and in another experiment after 12 hours. In the last two cases the animals were given glucose before the fat meal. On the contrary the phosphatides of the corpuscles show only small and quite irregular changes. As to the acid-soluble P increases were found in all the experiments (in No. 1, the fasting value was lost), in some cases small indeed, but certainly exceeding the experimental error. In Exps. 2 and 3 the rise and fall are regular with a maximum 4 hours after fat ingestion. In the rest of the experiments the changes are more irregular but in no instance is there a significant fall below the fasting values. The level of the non-lipoid P of the corpuscles is not moved definitely in any direction. Small variations occur but in quite an irregular way. In all experiments the plasma became markedly lipaemic, the maximal milkiness appearing as a rule after 4 hours. In the three control experiments (XI–XIII, Table II) in which lean meat was given but no oil the phosphatides of the plasma remained constant but the acid-soluble P of the plasma showed a rise of about the same magnitude as in the experiments with fat ingestion. In the three control experiments (VIII–X, Table II), with no food supply the plasma phosphatides as well as the acid-soluble P were unchanged.

Table II. *Control experiments.*

Exp. No. Date Exp. animal	Hours	Plasma		Corpuscles
		Phosphatide P mg./100 cc.	Acid-soluble P mg./100 cc.	Phosphatide P mg./100 cc.
VIII	0	11·7	4·5	—
24. i. 1931. Dog 1. ♂	4	11·6	4·3	—
IX	0	11·4	3·4	—
15. ii. 1931. Dog 1. ♂	4	11·3	3·5	—
X	0	9·9	3·5	—
15. ii. 1931. Dog 2. ♂	4	10·0	3·6	—
XI	0	15·5	3·5	14·1
14. viii. 1930. Dog 3. ♀	5	15·2	5·2	13·6
XII	0	11·5	3·7	—
24. i. 1931. Dog 2. ♂	4	11·5	4·4	—
XIII	0	11·7	4·2	—
30. i. 1931. Dog 1. ♂	4	11·7	5·0	—

Discussion.

My experiments were arranged in much the same way as Bloor's experiments in which this author found a marked phosphatide increase in the corpuscles. Among the obvious differences entailed in the experiments, however, none can to my mind be presumably held responsible for the marked discrepancy found in the results. My dogs (with one exception) were given

some meat together with the oil, whereas Bloor's animals had oil only. In Exp. 6 in which I gave no meat the outcome is, however, quite the same as in the rest. I made my determinations directly on the corpuscles, whereas Bloor obtained his corpuscle values as differences between whole blood and plasma, guided by haematocrit determinations. Bloor's corpuscle values, therefore, contrary to mine, include the leucocyte phosphatides. Yet, not even a considerable digestive leucocytosis, in spite of the relative richness of the leucocytes in phosphatides [Boyd, 1931] could produce the difference. The facts that I drew the blood from a vein of a hind leg, whereas Bloor used blood from the jugular vein and that different analytical methods were employed cannot reasonably explain the different results. There are certain reasons for believing that the diet during the period preceding the experiments might have been of some influence. According to Wendt [1928] and to Rony and Ching [1930] the fatty acid increase in plasma is much higher in animals which have fasted for some days previous to the fat meal, than in animals fed in the usual way. Bang [1918], Rony and Ching [1930] and others have found that ingestion of carbohydrates together with fat tends to repress the alimentary lipaemia. On the other hand, our experiments in which glucose was given before the fat meal show practically the same results with regard to phosphatides and non-lipoid P as the rest. In two of the glucose experiments the phosphatide increase in plasma appears somewhat protracted, but in the third the course of the phosphatide level was quite the same as in the experiments without glucose. Bloor does not mention anything about the diet used between the experiments. My animals received, as stated above, a mixed diet consisting mainly of meat. It remains to state that at present it is impossible to explain why a fat meal should be followed by a phosphatide increase in the corpuscles in some dogs and not in others.

After having found that in haemorrhagic lipaemia the phosphatide increase in the corpuscles is inconsiderable as compared with that in plasma, Bloor suggests that the absence of the rise in the corpuscles here and in certain cases of alimentary lipaemia may be due to the rapid passage of lecithin from the corpuscles to the plasma. Yet if this assumption were right, the new formation of lecithin in the corpuscles would be expected to cause a decrease of the non-lipoid P of the corpuscles and eventually of the plasma, especially when the new formation of lecithin had been going on for some time, that is towards the end of fat absorption. In our experiments, however, the non-lipoid P shows in no instance the least tendency to fall. On the contrary, in the plasma the non-lipoid P shows some increase. (This increase could possibly be ascribed to the meat given, as it was only in the control experiments with meat that a similar rise was found. As in Exp. 6, where only oil was given and no meat, an increase was also found, the fat may be at least partly responsible for the rise in the rest of the experiments.)

My results are thus clearly against the view of a phosphatide formation within the corpuscles during fat absorption. They cannot, however, be said

definitely to disprove it. To settle this question qualitative investigations of the corpuscle phosphatides during fat absorption will no doubt also be necessary.

SUMMARY.

1. In experiments on dogs the phosphatides of plasma showed after fat ingestion a regular increase, whereas the phosphatides of the corpuscles remained practically unchanged.

2. The non-lipoid P of plasma showed a small increase during fat absorption, the non-lipoid P of the corpuscles being unaltered.

3. The results argue against the assumption that the fat absorbed from the intestine is transformed into phosphatides of the red corpuscles of the blood.

The writer wishes to thank Prof. G. Blix for suggestions and advice in the work.

REFERENCES.

Bang (1918). *Biochem. Z.* **91**, 111.
Bloor (1915). *J. Biol. Chem.* **23**, 317.
—— (1916). *J. Biol. Chem.* **24**, 447.
—— (1920). *J. Biol. Chem.* **45**, 180.
Boyd (1931). *J. Biol. Chem.* **91**, 1.
Hiller, Linder, Lundsgaard and Van Slyke (1924). *J. Exp. Med.* **39**, 931.
Iwatsuru (1926). *Pflüger's Arch.* **214**, 295.
Knudson (1917). *J. Biol. Chem.* **32**, 337.
McClure and Huntzinger (1928). *J. Biol. Chem.* **76**, 1.
Page, Pasternack and Burt (1930). *Biochem. Z.* **223**, 445.
Rony and Ching (1930). *Endocrinology*, **14**, 355.
Wendt (1928). *Klin. Woch.* **7** (II), 2183.
Widmark and Vahlquist (1931). *Biochem. Z.* **230**, 245.

CLXXV. THE DETERMINATION OF TRYPTOPHAN AND TYROSINE.

By GLADYS ANNE DA SILVA.

From the Biochemical Department, King Edward VII College of Medicine, Singapore.

(*Received August 17th, 1931.*)

The colorimetric estimation of tryptophan and tyrosine, published by Folin and Ciocalteu [1927], has been used lately by several workers. Marston [1928] determined the amounts of these amino-acids in keratin from sheep's wool and considered the determinations to be satisfactory. Hanke [1928] found that the method could not be used for the estimation of tyrosine in the presence of cystine. He noted further, that a crude protein hydrolysate, either acid or alkaline, appeared to contain something other than tyrosine and tryptophan which interferes with the determination of these amino-acids. McFarlane and Fulmer [1930], using the method for the estimation of these amino-acids in some food proteins, experienced similar difficulties. More recently, Carpenter [1931] has used the method in determining the molecular weight of caseinogen. He does not appear to have recognised any difficulty except that he observes that only 81·3 % of added tryptophan was recovered. He estimated tryptophan, not only colorimetrically, but also by bromination.

This latter modification has been subjected to an investigation in this laboratory for the past two years which seems to demonstrate some important difficulties which are likely to be encountered in carrying out the procedure. The work was undertaken as part of an investigation into certain discrepancies in the methods of protein analysis, particularly with reference to the Van Slyke method. It was found that the decomposition products of tryptophan are likely to become involved, so that it was desirable to use a rapid and simple method of estimating tryptophan. As will be shown, this object has not altogether been achieved, but when using this procedure it is necessary to take into account the presence of histidine. The method appears to offer as good an estimation of histidine as it does of tryptophan. The main part of the investigation will form the subject of a further communication, but in view of a somewhat general adoption of the method of estimating tryptophan and tyrosine, it is thought that publication of these experiments, at this time, may be of interest.

Experimental.

Plimmer and his associates [1913, 1924] have shown that bromine may be absorbed by tryptophan, tyrosine, histidine, and cystine. Homer [1915] obtained slightly higher results for tryptophan.

Two cc. of a 1 % solution of each amino-acid (except tyrosine) were used for each determination, and were brominated according to the procedure of Plimmer and Phillips [1924], using 10 cc. of 0·2 *N* sodium bromate, 10 cc. of a 20 % solution of sodium bromide, and 5 cc. conc. HCl. After standing for 15 minutes, 10 cc. of a 4 % solution of sodium iodide were added and the liberated iodine was titrated with standard sodium thiosulphate. The thiosulphate solutions were carefully standardised by potassium dichromate, and frequent blank estimations were carried out with the above reagents.

Tryptophan.

cc. thiosulphate used	g. bromine absorbed
7·7	0·05760
7·9	0·05504
7·7	0·05760
7·6	0·05888

Blank experiments required 12·2 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds closely to an absorption of 7 atoms of bromine by 1 molecule of tryptophan.

Tyrosine. 5 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
7·1	0·06784
7·2	0·06656
6·7	0·07296
7·1	0·06784

Blank experiments required 12·4 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds to an absorption of 3 atoms of bromine by the molecule of tyrosine—a value, higher than that given by Plimmer and Phillips.

Histidine. 2 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
17·9	0·020185
17·9	0·020185
*17·3	0·025029

* This experiment was allowed to stand for 30 minutes.

Blank experiments required 20·4 cc. thiosulphate.

1 cc. thiosulphate = 0·008074 g. bromine.

This corresponds to an absorption of 2 atoms of bromine by 1 molecule of histidine.

Cystine. 1 cc. of a 1 % solution was used for each determination.

cc. thiosulphate used	g. bromine absorbed
9·85	0·03136
9·80	0·03072
9·85	0·03136

Blank experiments required 12·2 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds to an absorption of 10 atoms of bromine by the molecule of cystine.

Hydrolysis and precipitation of the proteins.

Hydrolysis has been carried out for 20–24 hours with 20 % NaOH in such quantity that 4 g. NaOH were present for each g. of protein. Two cc. of butyl alcohol were added. At the end of the period the condenser was removed and the butyl alcohol driven off. Sufficient 14*N* H_2SO_4 to neutralise the NaOH was added in accordance with the directions of Folin and Ciocalteu [1927], and after shaking, the flask was allowed to cool. The solution was then made approximately *N* with respect to H_2SO_4. Filtering was carried out using a fairly large funnel (11 cm. diameter). The precipitate and paper were thoroughly washed in a beaker with 7*N* H_2SO_4. The washings were added to the solution and the final volume was made up to 100 cc. It was found that a Jena flask gave considerably less residue than a Pyrex flask, and accordingly less nitrogen was lost by adsorption.

Before precipitating, aliquot portions of this solution were titrated, and the bulk was made *N* with respect to H_2SO_4. Quantities of 8 cc. were placed in centrifuge tubes, precipitated with 15 % $HgSO_4$, washed, and decomposed in accordance with the procedure of Folin and Ciocalteu. The precipitate and the filtrate portions were made up to suitable volume and the determinations carried out on aliquot portions. Each experiment was carried out at least in duplicate.

Experiment with pure amino-acids.

A mixture containing 15·198 mg. histidine, 120·2 mg. tyrosine, 113·4 mg. tryptophan, and 167·0 mg. cystine was hydrolysed with NaOH in accordance with the above procedure. The precipitate portion was examined for total nitrogen, amino-nitrogen, using Van Slyke's apparatus, and for bromine absorption.

	mg.
Total nitrogen found	= 19·631
Calculated for 113·4 mg. tryptophan	= 15·588
Difference	+ 4·043
Amino-nitrogen found	= 9·139
Calculated for 113·4 mg. tryptophan	= 7·794
Difference	+ 1·345

Since the extra amino-nitrogen is approximately one-third of the extra total nitrogen, and is therefore in the same proportion as in histidine, it appears probable that histidine has been precipitated with the tryptophan under the conditions of experiment. It may be recalled that precipitation with $HgSO_4$ in 5 % H_2SO_4 is a regular procedure for the isolation of histidine [see also,

Onslow, 1921]. If histidine and tryptophan alone are present, the amount of histidine-nitrogen may be calculated from the formula

$$\text{Histidine-N} = 3 \times \text{total N} - 6 \times \text{amino-N}.$$

Applying this to the above figures

	mg.
Histidine-N	= 4·059
Calculated for 15·198 mg. histidine	= 4·088
	− 0·029

which would represent a loss of 0·7 %.

	mg.
The amount of bromine absorbed by this solution	= 254·08
Calculated for histidine + tryptophan	= 282·12
	− 28·04

This represents a loss of about 9·9 % on the combined values for histidine and tryptophan, and 11·6 % if the loss is merely attributed to the tryptophan. The experiment has shown that in the presence of histidine, the tryptophan may not be estimated accurately, but on account of the nitrogen figures it does not appear that either tyrosine or cystine has contributed to the error.

When the filtrate from the mercuric sulphate precipitate was examined, loss was found to have occurred.

	mg.
The amount of bromine absorbed	= 353·33
Calculated for tyrosine + cystine	= 714·72
	− 361·39
Total nitrogen found	= 29·731
Calculated	= 31·355
	− 1·624

only 49·4 % of the calculated bromine absorption and 94·8 % of the total nitrogen have been secured. If we assume that the loss is entirely due to cystine, and calculate accordingly, we find that the bromination represents a loss of 65 % cystine, although 93 % of the cystine-nitrogen is still present. Consequently, therefore, there is the probability that some cystine has escaped destruction, and may, as pointed out by Hanke [1928], interfere with the estimation of tyrosine. This interference would be experienced with bromination methods just as much as with colorimetric methods as cystine absorbs rather a large quantity of bromine (10 atoms). It will be shown that the extent of the destruction of cystine by alkali is variable.

On acidifying the alkaline hydrolysate, a strong smell of H_2S may be obtained, if the amount of cystine originally present is large. Cystine has therefore been hydrolysed by the above procedure, and directly after acidifying the hydrolysate, attempts have been made to estimate the H_2S by sucking it

through a solution of cadmium chloride and weighing the CdS formed. In one experiment where 0·2132 g. cystine was used, an amount of CdS equivalent to 0·0629 g. cystine was weighed, which represents 29·5 %. In another case using 0·2524 g. cystine, an equivalent of 0·02749 g. was recovered—a loss of 10·8 %. On another occasion the hydrolysis was continued for 100 hours and no CdS was obtained.

Continuing the usual procedure of precipitating with $HgSO_4$, bromination could be carried out with the filtrate, but no bromination was found to take place with the precipitate portion. In the above three experiments with cystine 2·8 %, 10·5 % and 5·2 % cystine have been accounted for by bromination. The results with cystine are thus very variable.

Experiments with proteins.

In view of the above results with pure amino-acids, it was of interest to carry out the procedure with a protein from which tryptophan was absent. Gelatin was selected and 1·0492 g. was hydrolysed as above, and the procedure of precipitating with $HgSO_4$ carried out.

Total N of the precipitated portion = 1·3737 mg., which if calculated as histidine = 0·5 %. Amino-N = 0·4691 mg., which equals 0·49 % histidine in gelatin, and the bromine absorption of 1 cc. = 0·0612 mg., which represents 0·46 % histidine present. Rosedale (unpublished data) has carried out a series of determinations by Van Slyke's method on this sample of gelatin and finds the value for histidine to be 0·55 %.

In another experiment tryptophan (0·0956 g.) was added to gelatin (0·8632 g.). The procedure was identical with the experiment above, and the results from the precipitate portion were:

	mg.
Total N =	14·237
Amino-N =	6·934

From the previous experiment with gelatin alone, 4·92 mg. histidine were calculated, = 1·114 mg. histidine-N. Applying the formula used in the first experiment, we get 1·107 mg. histidine-N giving a loss of 0·007 mg., which represents 0·7 %.

Bromination showed an absorption of 2·823 mg. bromine. Allowing for 5·072 mg. the amount calculated for 4·92 mg. histidine, this represents 78·588 mg. tryptophan present, which is 82·2 % of the amount originally added, and is in agreement with the experiments of Carpenter [1931].

A quantity of 0·9886 g. caseinogen was treated similarly, and the following results were obtained from the precipitate portion:

	mg.
Total N	= 9·7342
Amino-N	= 3·4626
Bromine absorbed	= 56·600

If the histidine is calculated by the formula from the nitrogen figures, we find 31·3 mg., which represents 3·1 % histidine in caseinogen. In the remainder, 1·3072 mg. tryptophan-nitrogen are present, representing 0·9 % of tryptophan in caseinogen, a figure which is low even when compared with results obtained by isolation. According to the bromination, if we allow for the calculated amount of histidine, we find only 0·86 % tryptophan. If on the other hand the presence of histidine were ignored, the bromination would give 2 % tryptophan.

It seems doubtful, however, whether tryptophan may be estimated from the nitrogen figures without taking the histidine into account, and even then it appears that low results are likely to be obtained.

Folin and Ciocalteu did not attempt to estimate tryptophan by bromine absorption and the question arises as to whether the interference of histidine might not be avoided by the use of the colorimetric determination. No series of such determinations has been made in the present work, partly because it seems evident that some of the tryptophan has been destroyed in the process of hydrolysis, and partly on account of the fact that it was found that histidine dihydrochloride imparted to the phenol reagent a greenish tint which it was considered would cause too high a reading to be made for tryptophan. The addition of sodium cyanide did not prevent this, so that any further attempt to use this method was abandoned.

Conclusions.

If tryptophan is to be estimated after alkaline hydrolysis and precipitation with $HgSO_4$, histidine, which is precipitated at the same time, must be taken into consideration. It appears that this may best be done by carrying out determinations of total nitrogen and amino-nitrogen at the same time that the colorimetric or bromination estimations are made. If the histidine is thus allowed for, it appears that the amount of tryptophan present in the solution at the time may be determined. From the results with caseinogen, it seems evident that tryptophan may be lost owing to decomposition during hydrolysis. Onslow [1921] has used baryta as hydrolytic agent, but the difficulty in applying this to such small quantities of material is that, on acidifying before precipitation with $HgSO_4$, a relatively large precipitate of $BaSO_4$ would be formed and would be difficult to wash. Onslow points out that after hydrolysis with baryta peptides are found in the mercury precipitate. This seems to have been avoided in this instance owing to the use of strong alkali. Accurate estimations of tryptophan in a protein appear to require application of Onslow's method upon larger quantities of material.

Summary.

1. An attempt has been made to estimate tryptophan and tyrosine by bromination after alkaline hydrolysis and separation by $HgSO_4$.

2. Any cystine which may escape decomposition by hydrolysis interferes with the estimation of tyrosine.

3. Histidine appears to be precipitated with tryptophan and must be taken into account. A method of doing this by means of nitrogen determinations is suggested.

In conclusion, I desire to thank Prof. Rosedale, for his interest in the work, and for much help during its progress.

REFERENCES.

Carpenter (1931). *J. Amer. Chem. Soc.* **53**, 1812.
Folin and Ciocalteu (1927). *J. Biol. Chem.* **73**, 627.
Hanke (1928). *J. Biol. Chem.* **79**, 587.
Homer (1915). *J. Biol. Chem.* **22**, 369.
Marston (1928). *Council for Sci. and Ind. Res. (Australia), Bull.* 38.
McFarlane and Fulmer (1930). *Biochem. J.* **24**, 1601.
Onslow (1921). *Biochem. J.* **15**, 383, 392.
Plimmer and Eaves (1913). *Biochem. J.* **7**, 297.
—— and Phillips (1924). *Biochem. J.* **18**, 312.

CLXXVI. EFFECT OF BACTERIOPHAGE ON THE OXIDATION-REDUCTION POTENTIALS OF *B. COLI COMMUNIS* CULTURES.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories, London County Council, Sutton, Surrey.

(*Received August 15th, 1931.*)

Addition of bacteriophage to cultures of *B. dysenteriae* (Shiga) has the effect of inhibiting the fall in potential associated with the growth and metabolic activities of the organisms [Hewitt, 1931, 1]. The oxidation-reduction potentials developed in cultures of *B. coli communis* and the effect thereon of bacteriophage are described in this communication.

Methods.

The general methods and apparatus used have been described previously [Hewitt, 1931, 2]. In each experiment 8 cc. of culture medium were inoculated with 0·1 cc. of a 24-hour broth culture of *B. coli*. Dr C. H. Andrewes kindly provided a sample of bacteriophage and a culture of *B. coli communis* which was sensitive to the action of the bacteriophage. Before use the bacteriophage was subjected to several passages by adding it to 6-hour broth cultures of *B. coli* which were incubated for a further 18 hours and then filtered through small Seitz filters.

Results.

Cultures without bacteriophage.

Plain broth cultures. In peptone-infusion broth the potential of stationary aerobic cultures of *B. coli* fell rapidly, reaching its lowest level, E_h − 0·28 v., in $3\frac{1}{2}$ hours. The low level of potential reached is probably due to liberation of hydrogen, visible liberation of gas in the cultures being observed after about 4 hours' incubation.

In vigorously aerated broth cultures there was a slightly longer lag period before the potential began to fall rapidly, but the lowest level of potential was reached in $3\frac{1}{2}$ hours as with the stationary aerobic culture. The lowest level of potential noted in the cultures examined (− 0·36 v.) was somewhat lower than in stationary aerobic cultures. In cultures at a low potential, in which hydrogen liberation would be expected, the potential was observed to vary during the pulse of the aeration apparatus. The aeration apparatus was working at the rate of 10 cycles per minute and the potential rose during each

aeration stroke and then gradually fell again until the next stroke. The fluctuation varied from a few millivolts to nearly 0·1 v. Presumably hydrogen was being liberated rapidly and during each aeration stroke a portion of the hydrogen in the culture was displaced by air. The fact that as low a potential as − 0·36 v. could be established in a vigorously aerated culture illustrates the intense metabolic and enzymic activity displayed by *B. coli communis*.

Since *B. coli* contains catalase it was not surprising to find that no evidence could be obtained of the establishment of the high potentials corresponding to peroxide formation.

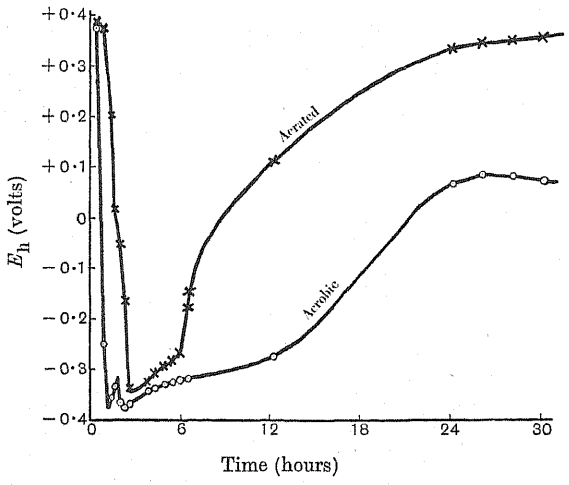


Fig. 1. Glucose broth cultures of *B. coli*.

Glucose broth cultures (Fig. 1). The fall in potential in 1 % glucose broth cultures was the most rapid yet recorded. In stationary aerobic cultures the potential had reached its lowest level (− 0·38 v.) in 2 hours. The most rapid fall in potential observed was 0·65 v. in 30 minutes. The kink in the potential-time curve which occurred in this case after 1 hour's incubation has been observed also with haemolytic streptococci.

In aerated glucose broth cultures the fall in potential was only slightly less rapid than in stationary aerobic glucose broth cultures, but after the logarithmic phase of growth the potential rose very rapidly.

Effect of bacteriophage on potentials.

0·1 cc. or 0·25 cc. of bacteriophage was added to each cell containing 8 cc. of culture.

Plain broth cultures. In stationary aerobic broth cultures containing 0·1 cc. of bacteriophage (Fig. 2) the potential fell to the level + 0·05 v. where it was maintained for some hours. The potential then began to fall again and

after 24 hours' incubation the low level reached in cultures without bacteriophage had been attained.

In aerated broth cultures containing 0·1 cc. of bacteriophage (Fig. 3) there was a delay of 6 or 7 hours before the potential fell below the level + 0·2 v. but after 12 hours' incubation the potential had reached almost as low a level as in aerated cultures not containing bacteriophage.

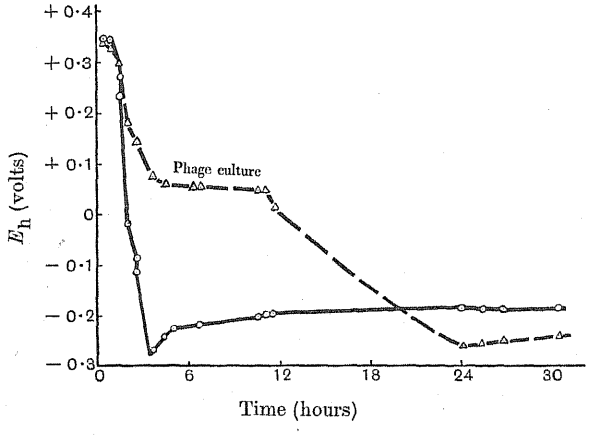


Fig. 2. Aerobic broth cultures.

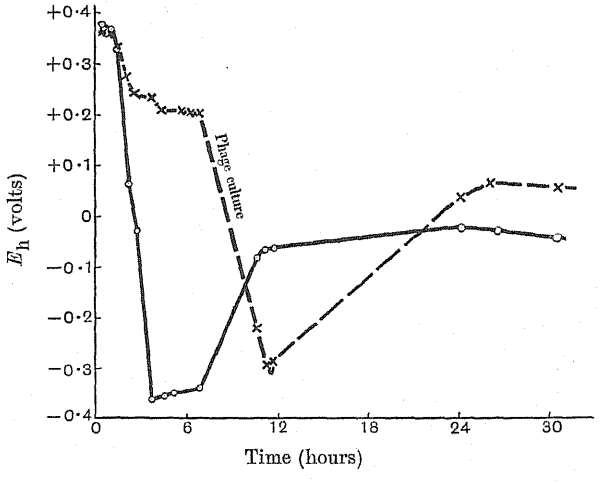


Fig. 3. Aerated broth cultures.

Glucose broth cultures. In stationary aerobic 1 % glucose broth cultures containing 0·25 cc. of bacteriophage (Fig. 4) the initial fall in potential was as rapid as in cultures without bacteriophage, but after this initial fall to a low level there was a rapid rise of potential. Later there was a further fall in potential followed by another rise and fall. Both growth and gas formation are visible in 6-hour glucose broth cultures to which bacteriophage has not

been added, but in cultures containing bacteriophage neither growth nor gas formation is observed until much later (see p. 1646).

In aerated glucose broth cultures containing bacteriophage (Fig. 5) the initial fall in potential was comparatively slight and not as rapid as in cultures without bacteriophage. The initial fall in potential was followed by a rise

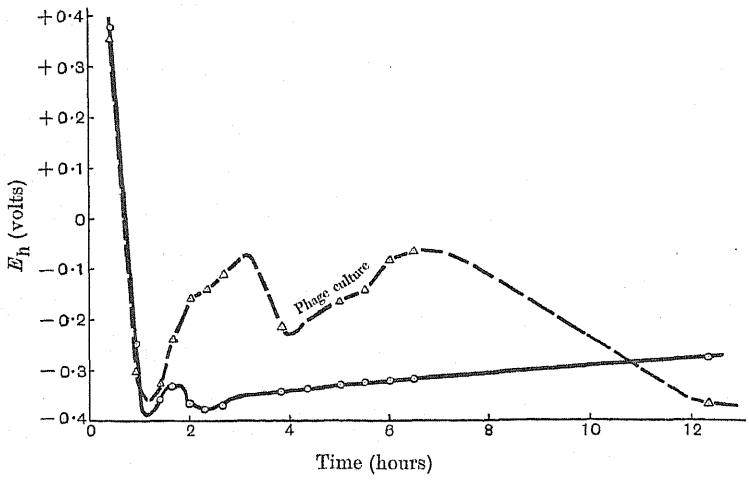


Fig. 4. Aerobic glucose broth cultures.

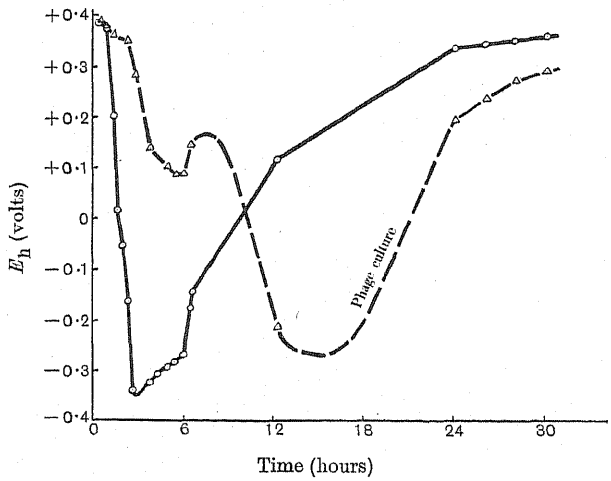


Fig. 5. Aerated glucose broth cultures.

and a subsequent fall to a low level when growth in the culture became visible. The reducing activity of the non-proliferating organisms was not sufficient to overcome the oxidising effect of the extra oxygen supplied to the culture by means of the aeration apparatus, but subsequently, when the organisms had proliferated sufficiently to produce visible growth in the culture, the usual fall in potential occurred.

Discussion.

The electrode potential in cultures of *B. coli* attains lower levels than have been described with other bacteria in this series of communications. These low potentials are associated with the mechanism possessed by *B. coli* for the liberation of hydrogen in its cultures. It may be noted that the potential produced at an electrode in the presence of one atmosphere pressure of hydrogen at p_H 7·0 is − 0·43 v. (at 37°) and in the present series of observations a potential of − 0·36 v. was observed in a culture subjected to aeration. In addition to the attainment of low levels of potential the rate of fall of potential is very rapid in *B. coli* cultures. In 1 % glucose broth cultures the fall is particularly rapid and the minimum level of potential (*ca.* − 0·37 v.) is reached in just over an hour.

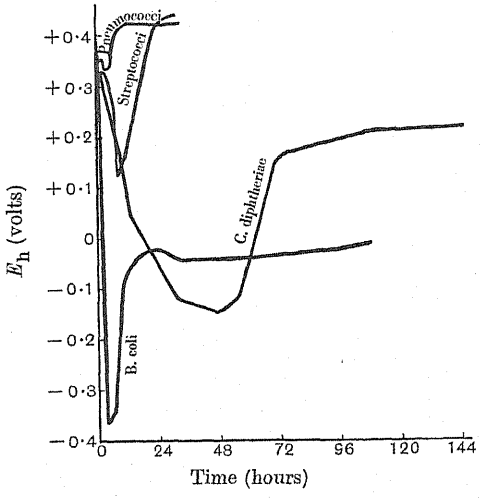


Fig. 6. Aerated broth cultures.

The characteristic behaviour of *B. coli* can be seen in Fig. 6 which gives the electrode potential time curves of aerated broth cultures of three classes of bacteria. Peroxide-forming organisms are represented by a pneumococcus and haemolytic streptococcus, catalase-containing organisms by *C. diphtheriae* and gas-forming organisms by *B. coli*.

The appropriate bacteriophage almost completely inhibits the fall in potential of *B. dysenteriae* (Shiga) cultures [Hewitt, 1931, 1], but in the case of *B. coli* the fall in potential is merely delayed and the potential eventually reaches approximately the same low level in the presence or absence of bacteriophage. The same observation was made with regard to the effect on the growth of the cultures. In the presence of the samples of bacteriophage used in these experiments no growth of *B. dysenteriae* (Shiga) was observed, but with *B. coli* visible growth was merely delayed when bacteriophage had been added. The results reported support the view previously expressed in

the case of *B. dysenteriae* [Hewitt, 1931, 1] that bacteriophage appears to have no effect on the oxidation-reduction potential of cultures except that it inhibits the usual fall in potential accompanying the proliferation and metabolic activities of the bacteria.

An interesting phenomenon was observed in stationary aerobic glucose broth cultures to which bacteriophage had been added. Despite the presence of bacteriophage the potential fell initially as rapidly as in its absence although the growth of the organisms was delayed. After this initial fall the potential rose fairly rapidly but fell again when active proliferation of the bacteria occurred. It is evident that the enzymes present in the bacteria inoculated into the medium were able to activate the glucose present and to establish temporarily intense reducing conditions. The re-establishment of low levels of potential occurred only when the numbers of bacteria present had increased greatly. In aerated glucose broth cultures containing bacteriophage the activity of the bacterial enzymes was insufficient to produce a large initial fall in potential and intense reducing conditions were not established until visible growth had occurred in the culture.

The general effects of the appropriate bacteriophages on the potentials of cultures of *B. dysenteriae* (Shiga) and *B. coli* are similar except that in the case of the Shiga bacillus the bacteriophage completely inhibits growth and the fall in potential, whilst in the case of *B. coli* proliferation and the fall in potential are merely delayed.

Summary.

1. The potential of *B. coli* cultures falls to a lower level and much more rapidly, especially in the presence of glucose, than in the case of other bacteria investigated. This is attributed to liberation of hydrogen in the culture and to a generally intense enzyme activity.

2. In the presence of bacteriophage the fall in potential and also the proliferation of the bacteria are delayed but not completely inhibited as in the case of *B. dysenteriae* (Shiga).

3. The effect of bacteriophage on the oxidation-reduction potential follows the effect on the proliferation of the bacteria, except that in aerobic glucose broth cultures of *B. coli* containing bacteriophage there is an initial fall in potential without appreciable bacterial growth, possibly due to activation of glucose by the enzymes of non-proliferating bacteria.

The author is indebted to Dr C. H. Andrewes for the culture and bacteriophage used and to Dr R. G. White and Dr E. W. Todd for their help and interest.

References.

Hewitt (1931, 1). *Biochem. J.* **25**, 1447.

—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry. (London County Council.)

CLXXVII. THE INFLUENCE OF SUNLIGHT ON MILK.

By HUGH ROBINSON WHITEHEAD.

From the Dairy Research Institute, Massey Agricultural College, Palmerston North, New Zealand.

(*Received September 9th, 1931.*)

Lojander [1925] noticed that exposure to sunlight decreased the time required to decolorise methylene blue in milk containing bacteria. The present author [1930] showed that sunlight caused a decoloration of methylene blue in milk independently of bacteria, and produced evidence in support of the theory that the reaction was accompanied by an oxidation of some portion of the milk fat.

Hammer and Cordes [1920] and Frazier [1928] had previously observed that whole milk exposed to sunlight developed a "cardboard" or tallowy flavour due to the oxidation of the milk fat. From the evidence available it seems probable that the two phenomena are based on the same chemical reaction so far as the fat is concerned, and that the presence of methylene blue in milk exposed to sunlight merely results in a visual indication of the intensity with which the fat oxidation is taking place. The author has observed that milk samples, exposed to sunlight with or without methylene blue, develop the tallowy flavour described by the American workers, and since neither the tallowy flavour nor the reduction of methylene blue occurs in milk from which the fat has been removed, it is almost certain that the two happenings are dependent upon one reaction—an oxidation-reduction phenomenon. The reaction appeared to be of sufficient interest to warrant a more detailed examination.

It has been shown by Clark *et al.* [1928] that the potential induced on an electrode of a noble metal immersed in a solution indicates the reducing or oxidising tendency more precisely and over a greater range than do the changes in colour undergone by a small amount of dye (such as methylene blue). The potentiometric method was applied, therefore, in an attempt to follow more fully the changes which take place in milk exposed to sunlight. Gold electrodes were immersed in tubes of milk. KCl-agar bridges connected the milk sample with a quinhydrone half cell prepared according to Veibel [1923]. The E.M.F. values given by such complete cells were measured at intervals by means of a potentiometer, and the E_h values calculated from the readings were plotted against time intervals. The milk used for the deter-

mination of the effect of sunlight was fresh and contained so few bacteria that it gave no appreciable change in E_h value within 7 hours when kept in darkness at 37°. In order to prevent the fat from rising too rapidly in the milk, the electrodes were moved gently up and down with a pumping action before taking each reading on the potentiometer, but they were never withdrawn wholly from the milk during the course of the experiment.

Whole milk.

When whole milk was exposed to sunlight, considerable changes in E_h were soon observed coincident with the decoloration of methylene blue in

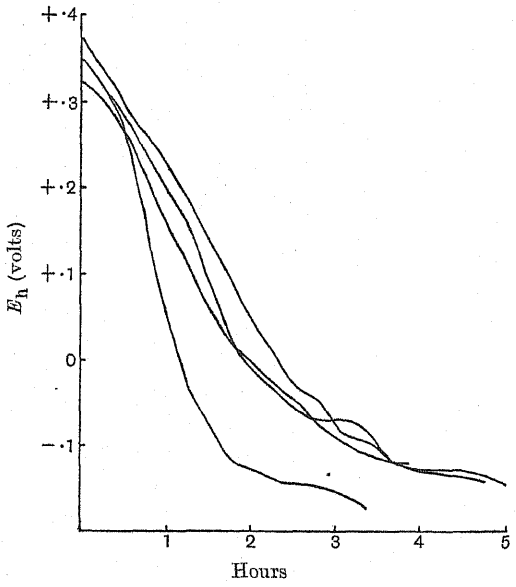


Fig. 1. Whole milk in bright sunlight.

duplicate samples also exposed to light. Fig. 1 shows the curves obtained in several typical experiments.

The fact that a fall in E_h occurs in the absence of methylene blue indicates that the dye is not essential to the reaction and that there must be some reversible system in milk capable of acting as a hydrogen acceptor. Clark and Cohen [1928] have shown that this is also the case in the Schardinger reaction. The time required to bring about the change and also the extent of the change varied considerably with different milks, with the intensity of the sunlight, and also with the particular electrode used. It soon became evident that the reducing potentials induced in the milk were very poorly poised and that any attempt to make accurate measurements would be useless. Different electrodes in duplicate samples of milk under apparently indentical conditions often showed differences of 50 millivolts in the potentiometer readings. The low

capacity of the reducing system formed during the fat oxidation was also evidenced by the fact that the presence of methylene blue (in dilution of 1 in 100,000) in milk in which the electrode was immersed resulted in a very definite tendency for the fall in potential to be checked in the region where methylene blue reduction occurs. In spite of this unavoidable inaccuracy, however, it was possible to gain some information from the general form of the curves which in a large number of experiments was fairly constant.

The form of curve obtained with whole milk exposed to sunlight is quite different from that obtained by plotting the changes of oxidation-reduction potential with time in milk in which bacteria are present in large numbers. In such cases the potential shows only a slight fall for some time and then drops very suddenly to a negative E_h value. The difference is easy to understand, for, when the action is due to bacteria, there is a rapid increase in the amount of enzyme (due to the multiplication of the organisms) during the course of the experiment, whereas in the action induced by sunlight there can be no increase in the amount of catalyst unless the intensity of the light increases markedly or the reaction is autocatalysed. The shape of the curve in the present experiments indicates that autocatalysis does not occur. In both types of reaction, however, the lower limit of E_h value reached appears to be about $-0{\cdot}2$ v.

An apparent anomaly in the course of the reaction which was often evident was that in bright sunlight the methylene blue underwent decoloration while the E_h of the milk was still over $+0{\cdot}2$ v., whereas the normal range for decoloration in aqueous solution is $+0{\cdot}07$ to $-0{\cdot}01$ v. at the p_H of milk. The reason for this has not been elucidated, but it seems very probable that, while the E_h of whole milk is changing rapidly, the potential on the electrode may lag in its indication of the conditions in the medium. This is especially likely to occur in the present case where the reaction responsible for the development of the reducing potential is taking place at the interface between two phases—the fat and the plasma. Some evidence that this lag in adjustment takes place was obtained by removing a tube of milk from bright sunlight to darkness midway in the reaction. When this was done, the potential on an electrode immersed in the milk continued to fall for some time, although it ceased to fall long before the potential in a duplicate tube exposed to sunlight the whole time. It seems more likely that in darkness the continued fall was due to an adjustment of the electrode to the actual conditions in the medium than that the reducing tendency continued to increase after the stimulus of sunlight was removed. Where the fall in potential was more gradual or less fat was present (as will be mentioned later), this decoloration of methylene blue at a potential apparently too high did not occur. In these cases also, removal of the tube from the influence of sunlight resulted in an immediate check in the fall in potential. It is suggested, therefore, that where the E_h falls more gradually or the medium is more nearly homogeneous, the potential on the electrode gives a more accurate indication of the conditions

in the medium from moment to moment. If this be the true explanation, it becomes evident that, under some conditions, a dye may give a truer indication of the oxidation-reduction potential of a solution than the potential on an electrode.

In early experiments the milk was maintained at 37° to 40° during exposure to sunlight. Owing to the inaccuracy of the potentiometric method, no attempt was made to determine the temperature coefficient of the reaction. It was found, however, that exposure of milk at room temperature resulted in a slowing of the reaction barely outside the limits of experimental error due to differences between electrodes. All later experiments were therefore performed at room temperature.

The reaction obtained with heated milk confirmed the results reported previously by the present author in that there was no essential difference from that obtained with raw milk.

Separated milk.

The results obtained by the exposure of separated milk to sunlight and measurement of the reducing potential were quite different from those obtained with whole milk and were not so regular. Many samples showed little change in E_h during the period of exposure to sunlight, this corresponding to the absence of change in methylene blue under such conditions. Other samples gave a decided fall similar to that obtained with whole milk, but the potential never fell much below zero E_h and then always showed a tendency to rise again to an E_h of between $+0{\cdot}2$ and $+0{\cdot}3$ v. This might be explained by assuming that the slight amount of residual fat (about 0·40 %) tended to undergo oxidation under the influence of the sunlight. The subsequent drift to a more positive potential can only be explained by assuming the presence in milk of some slowly reacting reducible system of large capacity, which in the oxidised form has an E_h of between $+0{\cdot}2$ and $+0{\cdot}3$ v. The presence of such a system in milk seems probable by reason of the fact that most milk samples, in the absence of influences such as sunlight and bacteria, attain equilibrium with a gold electrode at about this value of E_h. Fig. 2 gives several typical curves obtained with separated milk.

Although the curves obtained with some samples fell through the range where methylene blue reduction occurs, the dye was never reduced completely in separated milk. The reducing system had such a low capacity that the dye, when present, prevented by its poising action the extreme fall in E_h indicated in some of the curves. As has been mentioned before, a paling in colour sometimes took place with a subsequent deepening in colour as the potential rose again. The separated milk, moreover, never showed a continued fall in potential when the stimulus of sunlight was removed, and it is probable that the electrode was able to indicate the true potential of the medium more accurately from moment to moment. The occurrence or non-occurrence of a fall in potential in different batches of separated milk could not be correlated

with slight differences in fat content, and it is impossible at present to account satisfactorily for this variation in behaviour.

The effect of ultra-violet radiation.

Although in the foregoing experiments the sunlight passed through at least two layers of glass, the use of an ultra-violet spectroscope showed that some ultra-violet rays in sunlight were capable of reaching the milk.

In order to determine whether ultra-violet radiation was responsible for the stimulation of fat oxidation, experiments such as those already described were performed, replacing sunlight by radiation from a mercury vapour lamp. The tubes of milk were exposed at distances from the lamp varying from 1 ft.

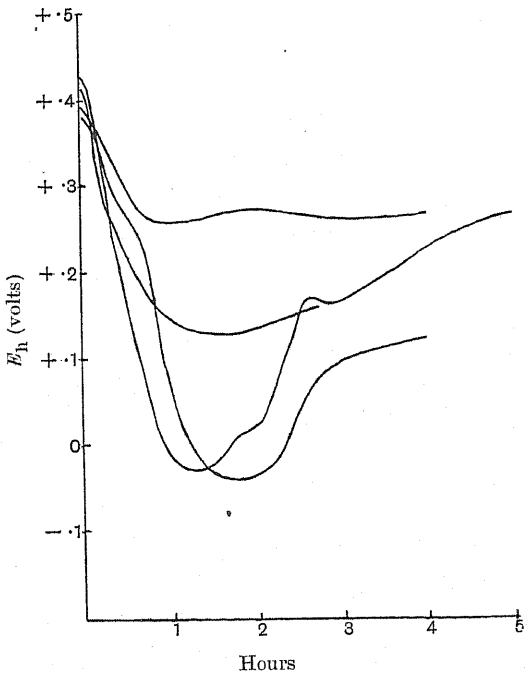


Fig. 2. Separated milk in bright sunlight.

to 6 ft. Even at the shortest distance, no change in blue colour was observed in milk containing the dye, and only a very slow change in potential was observed until about 6 hours had elapsed from the beginning of irradiation. It appeared to take at least three times as long to produce any effect by means of ultra-violet radiation as by sunlight. Another difference was that the blue did not begin to fade until the E_h had fallen below zero, and it seems most probable that, owing to the slowness of the changes taking place, the electrode was able to maintain its adjustment with conditions in the milk, thus indicating more exactly the true potential from moment to moment than was the case in milk exposed to sunlight and undergoing rapid changes. Separated

milk gave results similar to those obtained in sunlight, but again the course of any reaction which occurred was much more prolonged. The agent inducing oxidation of fat in milk is therefore not ultra-violet radiation (or at least any of the ultra-violet rays most characteristic of the radiation from a mercury vapour lamp), but more probably some portion of visible light of which there is less in the radiation of the mercury vapour lamp than in sunlight. Trials with radiation from electric lamps were negative so far as any effect on oxidation of fat or reduction of methylene blue were concerned, and at present it is therefore impossible to define more accurately the particular type of radiation responsible for the reaction.

Summary.

The oxidation of fat in whole milk under the influence of sunlight causes the development of a reducing potential, which can be detected either by its effect on methylene blue or by measurement electrometrically. The potential developed is poorly poised and of small capacity, hence experimental error in the measurement of potentials is great. Separated milk in many cases undergoes no change under the influence of sunlight, but some samples show a fall in potential which is not at present satisfactorily explained. When such a fall in potential occurs in separated milk there is a subsequent drift to more positive potentials. This suggests the presence in milk of a slowly acting reversible system which tends to maintain the E_h at a value between $+0{\cdot}2$ and $+0{\cdot}3$ v. It has not been found possible to reproduce the effect of sunlight by the use of either ultra-violet radiation or of radiation from electric lamps.

REFERENCES.

Clark *et al.* (1928). *U.S. Pub. Health Service, Hygenic Lab. Bull.* No. 151.
—— and Cohen (1928). *U.S. Pub. Health Service, Hygenic Lab. Bull.* No. 151, Section VIII.
Frazier (1928). *J. Dairy Science*, **11**, 375.
Hammer and Cordes (1920). *Research Bull.* No. 64, *Iowa Agric. Exp. Station.*
Lojander (1925). *Act. Soc. Med. Fennicae "Duodecim,"* 6, 1.
Veibel (1923). *J. Chem. Soc.* **123**, 2203.
Whitehead (1930). *Biochem. J.* **24**, 579.

CLXXVIII. A METHOD FOR THE DIRECT DETERMINATION OF UREA IN URINE.

By SYDNEY WILLIAM COLE.

From the Biochemical Laboratory, Cambridge.

(*Received August 27th, 1931.*)

The method introduced by Marshall [1913] is not used extensively, probably because of the length of time necessary for the completion of the action of the urease.

By using a stronger enzyme preparation and by maintaining the fluid under optimal conditions of reaction, it is possible to complete the estimation in about 15 minutes.

The principle of the method in brief is as follows. The urine is treated with warm water and some phenolphthalein, the urease preparation is added, and the mixture is titrated with 0·1 *N* HCl, at such a rate as just to discharge the red colour. This prevents the loss of ammonia and maintains a reaction of about p_H 8·2, which is about the optimum for urease acting on dilute solutions of urea. After about 10 minutes the mixture is treated with mercuric chloride and some methyl red. The addition of the HCl is continued until a red colour is obtained which matches that given by a mixture of the same amount of urine, mercuric chloride, enzyme preparation and methyl red which is titrated from a separate burette to give a red colour. In this case the mercuric chloride is added before the urease, which is unable to act in the presence of the mercury. A certain amount of ammonium chloride is added to the control to ensure uniform salt conditions for a correct adjustment of the final p_H. The difference between the amounts of HCl required for the two solutions is a measure of the urea in the urine taken.

Solutions required.

(*a*) 0·1 *N* HCl, in two burettes.
(*b*) Mercuric chloride. 0·5 % made up to 100 cc.
(*c*) Ammonium chloride, 10 %.
(*d*) Phenolphthalein. A saturated solution in 50 % alcohol.
(*e*) Methyl red. 0·02 % in 60 % alcohol.
(*f*) Urease solution. This is best made from "Arlco" Jack Bean Meal[1].

The simplest method is to place 4 g. of the meal in a mortar and rub it up

[1] This is prepared by The Arlington Chemical Co., Yonkers, N.Y., and is stocked by Baird and Tatlock, 14 Cross St., E.C. 1.

into a cream with 10 cc. of distilled water. Wash this into a beaker with more water until 100 cc. have been added. Stir well and allow to stand for some minutes. Pour off from a deposit of starch and other insoluble material. Such a preparation gives a considerable precipitate at the final end-point of the titration, but this does not materially interfere with the accuracy. The following method gives an almost clear solution. A 4 % suspension of the meal is made as above with the exception that the last 50 cc. of water are warmed to about 60° before being added. The whole is treated with 4 cc. of 0·1 *N* HCl, mixed well and filtered through a pleated paper. A water-clear filtrate is obtained. This gradually deposits a slight precipitate, which should not be filtered off.

Method of estimation. Into two similar 100 cc. beakers, labelled *A* and *B*, measure 3 cc. of the filtered urine. To each add about 20 cc. of distilled water at about 60° and 5 drops of the phenolphthalein. To *A* add 10 cc. of the enzyme preparation. As soon as this goes red add some 0·1 *N* HCl from a burette, and continue to do this at intervals, so as just to prevent a red colour developing. Care should be taken not to add too much acid, as this inhibits the action of the enzyme. As a rule it will not be necessary to add any acid after about 5 minutes. It is, however, advisable to allow the mixture to stand for about 12 or 15 minutes, depending on the concentration of the urea. During the interval place 1 cc. of the mercuric chloride and 1 cc. of the ammonium chloride in *B*. Mix well and then add 10 cc. of the enzyme preparation. Now add 20 drops of the methyl red (measured with a dropping pipette). With most samples of urine the mixture is yellow. Add 0·1 *N* HCl from a second burette until a full red colour is obtained, enough water being added to make the volume slightly greater than that in *A*. At the end of 12 or 15 minutes' digestion the fluid in *A* is treated with 1 cc. of the mercuric chloride, and 20 drops of methyl red. The contents of *A* are then titrated with the HCl until the colours of the fluids in the two beakers match, the volumes being kept constant by the addition of water to one or the other. It will be found that the red colour produced in *A* by the addition of acid tends to fade a little at first, owing to the rather slow establishment of equilibrium of the carbonic acid, but the two colours can be matched with great exactitude if a little care is given to this point. It is advisable to add enough acid to *B* to produce a rather bright red tint, otherwise the result may be a trifle low, owing to the effect of the carbonic acid in *A*. But the effect of this is negligible if the end-point of the titration is sufficiently acid.

Calculation. As 1 cc. of 0·1 *N* HCl is equivalent to 3 mg. of urea, the number of cc. of the acid added to *A* less that added to *B* gives the urea in g./litre.

Accuracy of the results. Duplicate estimations on the same sample of urine agree to 1 % with the filtered extract, and to about 2 % with the untreated suspension.

The results agree with those obtained by estimating the ammonia and the ammonia- *plus* urea-nitrogen by Van Slyke's methods [Van Slyke and Cullen, 1914] with the same degree of accuracy.

Theoretical results are obtained with solutions of urea up to 5 %, and also with urines to which urea has been added to make a total of 5 %.

With urines obtained when making the urea concentration test of renal efficiency, it is advisable to use 2 cc., and to multiply the cc. of acid used for *A* less that used for *B* by 1·5 to give the urea in g./litre.

SUMMARY.

A method is described for the estimation of urea in urine which can be completed in 20 minutes, and which is accurate to within 2 %.

REFERENCES.

Marshall (1913). *J. Biol. Chem.* **14**, 483.
Van Slyke and Cullen (1914). *J. Biol. Chem.* **19**, 141.

CLXXIX. THE ACCESSORY FACTOR NECESSARY FOR THE GROWTH OF *NEMATOSPORA GOSSYPII*.

I. THE CHEMICAL NATURE OF THE ACCESSORY FACTOR.

By HAROLD WILLIAM BUSTON and BIRENDRA NATH PRAMANIK.

From the Biochemical Department, Imperial College of Science and Technology, London, S.W.

(*Received August 20th, 1931.*)

Since the earliest attempts were made to cultivate fungi on synthetic nutrient media, cases have been met with in which certain fungi were unable to grow in such media in the absence of small amounts of extracts prepared from natural products. The best known instance of this difficulty of culture is that of ordinary yeast, *S. cerevisiae*, which has been shown to need the presence of a small quantity of "bios" to enable healthy growth to take place on salt-sugar media. The nature of "bios" remains undetermined, although the problem has engaged the attention of many workers for some 30 years.

In his studies on *Fusarium*, Brown [1925] devised a synthetic medium on which the growth of *F. fructigenum* was quite comparable with that on potato decoctions. This medium had the composition: glucose, 2 g.; asparagine, 2 g.; potassium phosphate, 1·25 g.; magnesium sulphate, 0·75 g.; agar, 15 g.; water, to 1000 cc. The same medium was found suitable for the cultivation of a large number of fungi, but cases were met with in which no growth could be obtained. Among the fungi unable to utilise the medium were certain varieties of *Nematospora*, including that investigated in the present instance, *N. gossypii*. The study of the nutritional needs of this organism was continued by Farries and Bell [1930], who were able to show that Brown's medium could be made entirely suitable if the asparagine were replaced by peptone or by the mixed hydrolysis products of certain proteins. They found further that the hydrolysis mixtures prepared from different proteins were not all equally suitable, those obtained from edestin and gelatin, for instance, being quite useless. Nor could the fungus utilise mixtures of all types of pure amino-acids, in the proportions in which they occur in a typical protein.

Farries and Bell concluded that, in general, the mixed hydrolysis products of purified proteins were valueless as a source of nitrogen for this fungus, whereas the hydrolysis mixtures from the crude proteins were entirely satisfactory, and gave good growth. They separated from egg-white, and also from

crude caseinogen, an "accessory factor," itself probably non-nitrogenous, the presence of which was essential for the utilisation of simple nitrogenous substances by *N. gossypii*. This "accessory factor" was readily obtained from egg-white by precipitation of the albumin with alcohol, the accessory being soluble in 80 % alcohol. Even the hydrolysis products of gelatin could readily be assimilated by the fungus in the presence of small amounts of this accessory.

The chemical nature of the accessory factor was not determined by Farries and Bell, although they made a number of experiments in an attempt to characterise the substance. They concluded that it was an organic compound, but not a polysaccharide, sugar, sugar alcohol or sugar acid, fat, ester or lipoid. They considered that it had acid properties, since it was precipitated by copper hydroxide and lead hydroxide. Whether the substance was nitrogenous could not be established; that it was not a basic amino-acid was shown by its non-precipitability by phosphotungstic acid or mercuric chloride. The most active concentrate prepared from egg-white by Farries and Bell had a very low nitrogen content.

It was in an attempt to gain more information as to the chemical nature of the "accessory factor" that the present work was undertaken.

The strain of fungus with which the experiments were carried out was that used by Farries and Bell, and the technique adopted was that which had been carefully standardised by these workers, and which is described briefly below. The use of egg-white as a source of the accessory factor was abandoned early in the work, for reasons given below, and, after a brief survey of other possible sources of the factor, it was decided to prepare the concentrates from lentils. Subsidiary tests were made with concentrates prepared from another plant source (lupin seedling extract) and from an animal source (ox-heart) and the results were found to be comparable. There was therefore no reason to suppose that egg-white would have proved any exception in respect of the nature of the accessory factor which it contained.

Experimental.

Method.

The organism used throughout the work was a strain of *N. gossypii* which had been isolated by Brown from diseased cotton bolls (East African). This strain gave vigorous growth, and when cultivated on solid media formed a tough mat of mycelium on the surface of the medium. The mat could be peeled off completely without disturbing the medium, and it was shown by Farries and Bell [1930] that the amount of mycelium remaining within the medium was only 3 % of the total mycelium. The "dry weight" method of estimating the growth of the fungus could therefore be adopted, the percentage error due to incomplete removal of the fungus from the medium being constant.

In order to ensure a maximum degree of regularity in the different cultures, the details of routine were standardised. The basal medium used had the following composition: glucose, 2 %; potassium phosphate, 0·5 %; magnesium

sulphate, 0·25 %; agar, 2 %. The nitrogenous constituent, from whatever source, was supplied in a concentration equivalent to 0·3 % N (see below).

The Petri dishes used were 3 in. in diameter, and the amount of medium added to each was 25 cc. A mycelium inoculation of the fungus (of which stock cultures were maintained on glucose-peptone agar) was placed at the centre of each plate, and the cultures were incubated at 30° for 6–7 days. At the end of this period the superficial mat of mycelium was peeled off the agar, dried on a tared filter-paper in a steam-oven for 48 hours and weighed. In general, the plates were treated in pairs, so that the "dry weight" figures quoted below represent the combined weight of mycelium from 2 plates.

As stated above, the nitrogenous constituent in the medium was supplied in a concentration equivalent to 0·3 % N. The main supply of nitrogen was provided by adding the appropriate amount of an amino-acid mixture prepared from gelatin by hydrolysing with 22 % sulphuric acid, removing excess of acid by baryta, and concentrating the filtrate to a known strength. Since the amounts of lentil and other concentrates tested themselves contained nitrogen, this was estimated and allowed for in adding the amino-acid mixture. In the tables given below, the term "standard basal medium" is intended to represent the salt-sugar mixture plus the 0·3 % N (from the combined sources, amino-acids and concentrate) referred to above.

The reaction of the medium was adjusted to p_H 6·8–7·0 before inoculation. The work of Farries and Bell had shown that the fungus possessed a comparatively wide range of growth, with an optimum at p_H 7·0.

In all cases where growth was found below normal, strict controls were set up to test for toxicity in the medium; this was particularly necessary, in view of the possibility of the presence of small traces of lead, barium, *etc.* in the media. In these cases, therefore, portions of the concentrate in question were added to a medium known to give good growth; if the resulting growth were at least as good as that on the standard medium, it was assumed that lack of accessory factor, and not toxicity in the concentrate, was responsible for lack of growth in the absence of the standard concentrate.

The effect upon growth of different amounts of concentrate was investigated, and it was found that addition of a small amount of concentrate produced a relatively large increment in growth, further small additions giving proportionate increases. A point was reached, however, after which the addition of a further large supply of concentrate produced only a small increment in growth, such as might often be accounted for by addition of actual food material present in the portion of concentrate added. If a curve were plotted showing the weight of concentrate added against the weight of mycelium produced, a fairly sharp change of gradient was observed where addition of more concentrate no longer produced great increase in growth. This point was taken as indicating the "critical amount" of the concentrate necessary and all comparisons of activity were made on the basis of this "critical amount" of concentrate. Examples of the method are given below.

Tests for activity in known substances.

Before attempting to fractionate the concentrates prepared from various sources, it was thought worth while to ascertain whether certain substances, which had from time to time been suggested as growth-promoting accessories by workers on animal nutrition and bacterial metabolism, could supply the essential nutrient for the growth of *N. gossypii*.

Nucleic acid (yeast), adenine sulphate, guanine hydrochloride, mannitol, inositol and inositol plus traces of Fe or Cu were tested, but in no case was any appreciable effect observed.

Sources of the "accessory factor."

As stated above, the material from which Farries and Bell prepared their active concentrate was egg-white, which they fractionated into "albumin" and "egg-extract" by precipitation with alcohol. They found that the albumin, whether hydrolysed or not, did not give any measurable growth of *N. gossypii*, but when supplemented with very small amounts of the "egg-extract" gave copious growth. According to their figures, the growth on albumin alone was 1·5 % of that on albumin plus "egg-extract." The first attempts to prepare concentrates of the active factor followed the methods given by Farries and Bell. It was found that the original egg-white (hydrolysed) gave good growth, and a sample of crystalline albumin prepared therefrom only negligible growth, thus confirming the results of Farries and Bell. On the other hand, the preparation of the "egg-extract" in good yield was found to be a matter of some difficulty, the active factor being retained somewhat tenaciously by the albumin when the latter was precipitated. On account of this difficulty in manipulation, and the expense involved in using egg-white as a starting material, other sources of the accessory factor were sought. At the same time, a number of substances were tested for the presence of the factor, so that a brief survey of the distribution of the factor among a variety of products might be obtained.

(*a*) *Cotton plant.* As the natural habitat of the fungus is in the bolls of the cotton plant, it was thought worth while to see whether other parts of the plant would also yield the accessory factor. Cotton leaves (about 50 g.) were ground to a pulp, and treated with 3 volumes of 95 % alcohol; the filtrate was evaporated, taken up in water and made up to 50 cc. Sufficient of this solution to correspond with 0·5 g. of solid matter was incorporated in 50 cc. of the medium and growth tested. The results of this and other tests on various extracts are summarised in Table I.

(*b*) *Potato.* 300 g. potato were steamed for 3 hours and the aqueous extract was precipitated with alcohol (3 volumes). The portion soluble in 75 % alcohol was taken up in water, and varying amounts were plated with the basal medium according to the usual procedure.

(*c*) *Lentils.* 300 g. portions of lentils were treated in the same way as the potatoes.

(*d*) *Lupin seedlings.* A portion of an extract of etiolated lupin seedlings which was available was treated with alcohol, as in the case of other extracts, to remove most of the inorganic salts, *etc.* The filtrate, after removal of alcohol, was tested for activity.

(*e*) *Yeast extract.* A sample of zymin was prepared from brewer's yeast by the acetone method. This was boiled with water for 30 minutes, and the solid material removed on the centrifuge. The extract was concentrated, and portions were incorporated in the basal medium. Similar tests were also made with a commercial yeast preparation (marmite), this being used without further treatment.

(*f*) *Ox-heart.* Preparations of ox-heart were used by Reader [1928] as a source of the factor promoting growth of *Streptothrix corallinus*; accordingly, a tryptic digest of fresh ox-heart was prepared according to the method described by Reader, except that a much longer period of digestion was found necessary to produce a mixture not giving the biuret reaction. The soluble portion of the resultant mixture was made up to a known concentration, and portions were added to the basal media.

(*g*) *Fibrin.* A sample of commercial fibrin (B.D.H.) was digested in acid solution with pepsin for 1 month. The insoluble residue was filtered off, and the filtrate tested for activity.

Table I.

Extract tested	Weight of concentrate in 50 cc. medium g.	Dry weight of mycelium (2 plates) g.
Cotton leaves	0·5	0·15
Potato	0·1	—
	0·2	0·05
	0·3	0·11
	0·4	0·14
	0·5	0·18
Lentils	0·1	0·28
	0·2	0·31
	0·3	0·33
	0·4	0·35
	0·5	0·375
Lupin	0·05*	0·06
	0·1	0·12
	0·2	0·195
	0·3	0·25
	0·4	0·295
	0·5	0·32
Yeast	0·05	0·08
	0·075	0·10
	0·1	0·13
(Marmite)	0·075	0·11
	0·15	0·185
Ox-heart digest	0·1*	0·08
	0·3	0·19
	0·5	0·25
Fibrin digest	0·1	—
	0·5	—

* Ash-free organic matter.

The relative activities of the concentrates prepared from different materials were clearly indicated by these tests. The greatest activity was shown by lentil extracts, while lupin extract, yeast and ox-heart were fairly rich in the factor. Potato was relatively poor, and fibrin deficient in the factor. (This deficiency was noted by Farries and Bell.) To the list of substances rich in the accessory must be added those recorded by Farries and Bell, *viz.* egg-white, impure caseinogen and commercial peptone.

In view of the results thus obtained, it was decided to use lentils as the starting material in attempts to prepare more active concentrates than those hitherto dealt with.

Preparation of the lentil concentrate.

About 50 kg. of lentils, dealt with in portions of 500 g., were steamed with twice their weight of water for 3 hours, the residual solid was removed, and the extract evaporated to about 300 cc. The addition of 3 volumes of alcohol gave a large precipitate, containing inorganic salts, proteins, *etc.* This was filtered off, and the filtrate again evaporated to a small bulk, and precipitated with alcohol. The filtrate at this stage was evaporated to a syrup and treated with dry acetone, thus granulating the substance. The solid was dried *in vacuo*, when it was obtained as a light brown, somewhat hygroscopic powder, containing 2·88 % N. The yield from 50 kg. of lentils amounted to about 100 g. Its activity was similar to that of the sample obtained on the small scale, as described above—0·1 g. in 50 cc. medium gave 0·28 g. dry mycelium.

When different amounts of the lentil preparation were incorporated in the medium, the corresponding growths obtained were those shown in Table II.

Table II.

Weight of lentil concentrate per 50 cc. medium g.	Dry weight of mycelium g.
0·01	—
0·025	0·06
0·05	0·13
0·075	0·195
0·10	0·28
0·20	0·31
0·30	0·33
0·40	0·35
0·50	0·375

It was evident from these results that the "critical dose" of the lentil concentrate lay in the region of 0·1 g. per 50 cc. of medium. Accordingly, all tests were made on a basis of 0·1 g. original concentrate per 50 cc. medium. Thus it was necessary at each stage of the fractionation to determine the weight of substance in each fraction, corresponding to 0·1 g. of the original substance; since there was a small loss of total substance at every stage of the fractionation process, it was assumed that the fractions (*e.g.* filtrate and ppt.) suffered the same proportionate loss.

Fractionation of the lentil concentrate.

In the course of their attempts to fractionate the "egg-extract," Farries and Bell found that the active factor was precipitated by copper hydroxide or lead hydroxide; accordingly, the first attempts to fractionate the lentil concentrate were made on these lines. It was found, however, that although the active substance was apparently precipitated by these reagents, it was impossible to recover it from the precipitates, probably on account of the fact that nearly all the active substance was adsorbed on the copper (or lead) sulphide produced when the precipitates were decomposed. For this reason these experiments were not proceeded with. A number of preliminary tests indicated that a considerable degree of concentration could be obtained by treatment of the lentil extract in the following manner.

(i) Removal of basic substances (inactive) by phosphotungstic acid.

(ii) Removal of the majority of organic acids as barium salts, by precipitation with baryta and alcohol.

(iii) Precipitation of the active factor as a mercury compound by Neuberg and Kerb's reagent (mercuric acetate and sodium carbonate).

Accordingly, 80 g. of the lentil preparation were submitted to the above process. The precipitation with phosphotungstic acid was carried out in the ordinary way, in the presence of 5 % sulphuric acid, which was subsequently removed from the filtrate by baryta. The activity was found to be confined to the filtrate, the basic substances being entirely inactive, even in high concentrations. From the 80 g. of original substance, 60·8 g. remained in the filtrate. The usual growth tests gave the following results:

Table III.

Medium	Dry weight of mycelium g.
Basal + 0·075 g. substance from phosphotungstic acid filtrate	0·245
Basal + 0·1 g. substance from phosphotungstic acid filtrate	0·315
Basal + 0·1 g. substance from phosphotungstic acid ppt.	—

A portion (30 g.) of the substance not precipitated by phosphotungstic acid was dissolved in 200 cc. of water, and the liquid was saturated with pure barium hydroxide; 3 volumes of alcohol were then added, and the mixture was kept at 0° overnight. The precipitate was washed with 75 % alcohol, and the precipitate and filtrate fractions were decomposed separately by means of sulphuric acid. The material recovered from the precipitate amounted to 21·6 g., and from the filtrate 5·6 g. The loss of material thus amounted to 2·8 g., and the proportion of precipitate fraction to filtrate fraction was almost exactly 4 : 1. Media were prepared containing the appropriate amounts of the two fractions and growth tested (Table IV). Since, as shown in the table, the activity had apparently been almost entirely lost, the usual toxicity tests were applied, but no evidence of the presence of any toxic substance could be obtained. Portions of the two fractions were then recombined, to reconstitute the original substance, when full activity was found to have been restored.

Table IV.

Medium	Dry weight of mycelium g.
Basal + 0·08 g. Ba ppt. fraction	0·02
Basal + 0·02 g. Ba filtrate fraction	0·018
Basal + 0·08 g. ppt. + 0·02 g. filtrate	0·285
Basal + 0·07 g. ppt. + 0·03 g. filtrate	0·265

These results gave a clear indication that the active substance present in the lentil concentrate could be fractionated into two substances, either of which alone was inactive, but in combination possessing the full activity of the original concentrate. By submitting the two fractions again to the precipitation, the activity of the separate fractions was further reduced, the amount of mycelium obtained being too small to allow of its being scraped from the surface of the medium. The process was repeated with a further small batch of the lentil concentrate (6 g.) and a similar result again obtained.

At this point it was realised that the results so far obtained—particularly the partition of the active factor into two inactive components—showed a close parallel with those reported by several observers on the "bios" of yeast. Eastcott [1928] claimed to have obtained two such fractions from a bios concentrate, and further stated that one of the factors was *i*-inositol (ppt. fraction). Although many subsequent workers had disputed her results [Narayanan, 1930] it was thought worth while to test the effect of inositol on the growth of *N. gossypii* in the presence of the baryta filtrate fraction. (Inositol alone had been found inactive in promoting growth—see above, p. 1659.) The results are given in Table V.

Table V.

Medium	Dry weight of mycelium g.
Basal + 0·02 g. Ba filtrate + 0·0005 g. inositol	0·13
,, ,, ,, + 0·001 g. ,,	0·15
,, ,, ,, + 0·005 g. ,,	0·19
,, ,, ,, + 0·007 g. ,,	0·245
,, ,, ,, + 0·01 g. ,,	0·315

Other media were tested in which the inositol was replaced, wholly or in part, by mannitol, but in no case was the presence of mannitol found to influence the amount of growth. The inositol itself was also carefully recrystallised several times, but again the repurified product did not differ in activity from the original sample (Kahlbaum). The evidence seemed conclusive that small amounts of inositol were able to replace the "baryta precipitate" fraction in the medium, in conjunction with the "baryta filtrate."

For this reason, the isolation of inositol from the baryta precipitate fraction was attempted. 20 g. of the fraction were dissolved in water, and the solution was treated with cold neutral lead acetate; after removal of the small precipitate, the liquid was treated with excess of basic lead acetate and ammonia, and allowed to stand overnight. The precipitate was removed, and decomposed with hydrogen sulphide in the presence of sulphuric acid; from the solution,

excess of sulphuric acid was quantitatively removed, and the liquid was concentrated to a small bulk. Five volumes of absolute alcohol and 1 volume of ether were added, and the whole was kept at 0° for 24 hours. The solid was removed and taken up in as little hot water (containing about 5 % nitric acid) as possible, and reprecipitated with alcohol and ether. After again taking up in hot water, the solution was treated with hot baryta solution; the filtrate was freed from barium with ammonium carbonate, evaporated to a small bulk, and precipitated with alcohol and ether. The precipitate was recrystallised from 50 % acetic acid, and after three recrystallisations was obtained as a white crystalline material, M.P. 223°. This contained[1] C, 40·4 %; H, 6·9 %. Calculated for inositol, $(CHOH)_6$, C, 40·0 %; H, 6·7 %. A mixed M.P. with a sample of Kahlbaum's inositol, recrystallised three times, showed no depression.

The hexa-acetyl derivative, prepared by the method of Müller [1907], had M.P. 214°; it showed no depression when mixed with a sample of hexa-acetyl-inositol prepared from Kahlbaum's inositol. It was therefore considered that the product isolated from the baryta precipitate fraction was inositol, which was known to be widely distributed among natural products. The amount isolated was 0·88 g. from 20 g. of the baryta precipitate fraction. A calculation of the amount present, based upon the relative activities of pure inositol and the precipitate fraction indicated the presence of about 2 g. of inositol in 20 g. of the fraction. The yield thus amounted to approximately 40 %, which was considered reasonable in view of the complexity of the process of separation.

Its growth-promoting activity was tested in the usual way, and was found to be the same as that of Kahlbaum inositol. 0·007 g. in conjunction with 0·02 g. of the baryta filtrate fraction in 50 cc. of medium gave 0·235 g. of mycelium (dry weight). The remainder of the baryta precipitate fraction, from which the inositol had been removed by the process described above, was carefully freed from lead, acetic acid, *etc.*, and portions tested for activity. It was found to be completely inactive and non-toxic, thus proving that the active factor had been entirely removed by precipitation with basic lead acetate. An experiment with pure inositol had shown that this substance was completely separated from a mixture with sugars by the same method. There seemed no doubt, therefore, that the substance responsible for the activity of the baryta precipitate fraction was inositol, and that no other active substance was present.

The baryta filtrate fraction.

So far, little has been done in attempts to isolate the active substance from the baryta filtrate fraction. Precipitation with the mercuric acetate reagent of Neuberg and Kerb was found to throw down the active substance, so that the possibility exists that it is associated with the amino-acids. That it is not one of the ordinarily occurring amino-acids follows from the fact that the hydrolysis mixture obtained from gelatin, supplemented with tryptophan,

[1] The micro-combustions were carried out by Dr J. A. B. Smith, to whom our thanks are due.

tyrosine and inositol was found unable to support the growth of *N. gossypii.* Investigations on this section of the work are still in progress.

Fractionation of concentrates from other sources.

The fractionation of the active factor into two components by means of baryta and alcohol in the case of lentils made it desirable to ascertain whether a similar result could be obtained when using concentrates prepared from other sources. Consequently, the following substances were investigated: peptone, tryptic digest of ox-heart, lupin extract. The method used was precisely the same as in the case of the lentil preparation, and the results obtained are given in Table VI.

Table VI.

Medium	Dry weight of mycelium g.
Basal + 0·12 g. Ba ppt. from peptone	—
,, + 0·27 g. Ba filtrate from peptone	—
,, + 0·12 g. ppt. + 0·27 g. filtrate from peptone ...	0·185
,, + 0·06 g. Ba ppt. from ox-heart	—
,, + 0·24 g. Ba filtrate from ox-heart	0·055
,, + 0·06 g. ppt. + 0·24 g. filtrate from ox-heart ...	0·195
,, + 0·07 g. Ba ppt. from lupin extract	—
,, + 0·03 g. Ba filtrate from lupin extract	—
,, + 0·07 g. ppt. + 0·03 g. filtrate from lupin extract	0·155

The results clearly indicated that the active factor present in each of these substances was split into two components, neither of which alone was active, but which in conjunction gave the normal degree of activity.

In the case of the lupin extract, inositol was actually isolated from the baryta precipitate fraction, the method being the same as that used in the experiments with lentils. The inositol-free residue was again shown to be inactive in combination with the baryta filtrate fraction.

Various experiments on the baryta precipitate fraction (inositol).

(*a*) *Hydrolysis with 25 % sulphuric acid.* Eastcott [1928] found that the "bios I" (inositol) content of her preparation, as judged by its "bios" activity, was trebled on boiling with 22 % hydrochloric acid for 24 hours. This she ascribed to the presence of a precursor of inositol, from which the latter was liberated by hydrolysis with acid. That no similar effect could be observed in the present case was proved by the following experiment. 25 g. of the lentil concentrate, after treatment with phosphotungstic acid, were hydrolysed with 25 % sulphuric acid for 24 hours. A large amount (3·2 g.) of insoluble matter separated. The solution after hydrolysis was freed from sulphuric acid in the usual manner, and the filtrates were evaporated. 15·8 g. of solid material were recovered, so that there was a loss of 6 g.; the loss was probably partly due to adsorption on the barium sulphate precipitates, and partly to destruction of sugars in the concentrate by the action of the strong acid. The activity of the product after hydrolysis was tested as usual (Table VII).

Table VII.

Medium	Dry weight of mycelium g.
Basal + 0·025 g. concentrate after hydrolysis	0·11
„ + 0·05 g. „ „ „	0·165
„ + 0·075 g. „ „ „	0·255
„ + 0·075 g. original concentrate	0·245

(*b*) *Recovery of the active factor from the mycelium.* In her work on "bios," Eastcott was able to recover from the yeast-crop grown on a medium containing a known amount of inositol the whole of the inositol supplied. Further, many workers have apparently shown that yeast manufactures "bios" during growth. It seemed therefore of interest to determine whether any similar effect could be traced in the case of *N. gossypii*. Extracts were prepared from the combined mycelia from 2, 4, 6, and 8 plates, and from the residual media after removal of the mycelia. Fresh media were prepared incorporating these extracts, allowance being made for both the nitrogen and the phosphate carried over in the extracts. In no case was the medium found able to support more than slight growth. If a large amount of extract of mycelium were used (1 g. in 50 cc. medium), the growth amounted only to 0·185 g. The evidence was, therefore, that far from producing the active factors during growth, the fungus largely used up the amount initially supplied in the medium. These results were thus in direct contrast to those noted by workers in the case of yeast and "bios."

(*c*) *Proportion of inositol to the baryta filtrate fraction giving optimum growth.* Since it had been proved that the accessory factor in the case of *N. gossypii* consisted actually of two substances, it seemed probable that the activity of an extract would depend not only on the total amount of the accessory factor present, but on the relative amounts of the two components, and that the high activity of such products as the lentil concentrate might be due to the presence of the two components in an optimum ratio. Experiments were made to see

Table VIII.

Medium	Dry weight of mycelium g.
Basal + 0·01 g. lentil extract	—
„ „ „ + 0·001 g. inositol	0·065
„ „ „ + 0·005 g. „	0·07
„ + 0·025 g. „	0·06
„ „ „ + 0·001 g. inositol	0·085
„ „ „ + 0·005 g. „	0·09
„ + 0·05 g. „	0·12
„ „ „ + 0·001 g. inositol	0·185
„ „ „ + 0·005 g. „	0·195
„ + 0·075 g. „	0·195
„ „ „ + 0·001 g. inositol	0·21
„ „ „ + 0·005 g. „	0·23
„ + 0·10 g. „	0·28
„ „ „ + 0·001 g. inositol	0·296
„ „ „ + 0·005 g. „	0·30
„ + 0·01 g. „ + 0·01 g. inositol	0·08
„ + 0·05 g. „ „ „	0·27
„ + 0·075 g. „ „ „	0·29
„ + 0·10 g. „ „ „	0·305

whether the addition of extra inositol to the lentil preparation gave any increased activity (Table VIII).

These results indicated that the activity of the lentil extract was somewhat increased by the addition of small amounts of inositol, the effect being most noticeable when dealing with small amounts of the concentrate. Relatively much less effect was observed when the medium contained larger amounts of the lentil preparation, suggesting that the fungus was unable to utilise amounts of inositol beyond a certain concentration.

The effect of adding inositol to marmite was also tested. As shown above (p. 1660), marmite was not found to be a particularly rich source of the accessory factor, but when supplemented with inositol, its activity was considerably increased. The conclusion was drawn that marmite was rich in the second factor but deficient in inositol.

Table IX.

Medium	Dry weight of mycelium g.
Basal + 0·075 g. marmite	0·11
„ „ „ + 0·01 g. inositol	0·24
„ + 0·15 g. marmite	0·185
„ „ „ + 0·01 g. inositol	0·31

(*d*) *Activity of derivatives of inositol.* In an attempt to ascertain whether the activity of inositol could be due to the presence of small amounts of active impurities, media were tested in which portions of the hexa-acetylinositol, prepared as described above, were incorporated. The derivative itself is insoluble in water, but an active impurity, unless itself converted into an insoluble acetyl compound, would be soluble and removed during acetylation. Further, the inositol obtained by hydrolysis of the acetyl compound should recover the full initial activity of the original inositol. On determining the activity of the derivative, it was found that the media containing the hexa-acetyl inositol supported a certain amount of growth (Table X). On further examining

Table X.

Medium	Dry weight of mycelium g.
Basal + 0·03 g. baryta filtrate + 0·025 g. hexacetyl inositol	0·225
Basal + 0·03 g. baryta filtrate + 0·007 g. inositol derived from hexacetyl compound...	0·285

this point, it was found that the acetyl compound was partly hydrolysed during the autoclaving of the medium; a loss of about 10 % occurred by autoclaving with water alone, and a hydrolysis amounting to some 80 % in the presence of small quantities of potassium phosphate. Thus the activity shown by the hexa-acetylinositol could be regarded as due to the liberation of free inositol in the medium. A test using a sample of inositol prepared from the hexa-acetyl derivative by hydrolysis gave the same amount of growth as given by the original inositol. If the activity of inositol were due actually to the presence

of an impurity, this impurity must be inseparable from inositol by acetylation, and the amount present too small to influence the ordinary chemical and physical properties of inositol and its hexa-acetate.

Discussion.

The experiments described above seemed to show conclusively that the accessory factor necessary for the growth of *N. gossypii* could be fractionated into two mutually complementary substances, one of which was shown to be inositol. The nature of the second factor was not determined; it was found to be precipitated with the monoamino-acids by Neuberg and Kerb's reagent, but it was not one of the amino-acids normally occurring among protein hydrolysis mixtures. Whether it was a single substance was not determined, but there was no evidence to the contrary.

What part is played by inositol in the metabolism of the fungus is entirely unknown. It is curious that this substance has been stated repeatedly to have some special significance in the promotion of growth or activity, and its isolation as a constituent of "bios" has been reported [Eastcott, 1928]. The view that inositol is concerned in "bios" activity is, however, by no means generally accepted. Thus Narayanan [1930], criticising the work of Eastcott, stated that "the activity of inositol must have been due either to the presence of an impurity, or to some such effect as has been described by Reader in the case of mannitol." (The following communication contains a further discussion of this point.)

In her observations on the effect of mannitol on bacterial growth, Reader [1929] used a medium in which 0·5 % glucose constituted the source of carbohydrate. To this was added a small amount (1/20 "unit dose" per 20 cc.) of torulin, and 0·5 % of mannitol. The resultant growth of *S. corallinus* showed a large increase (from 300 % to 600 %) over that given in the absence of mannitol. A similar effect, though much less pronounced, was given by citrate. When the concentrations of torulin and mannitol were reduced to 1/400th of their original amount, the excess growth due to mannitol was not observed. The explanation of these "anomalous results" put forward by Reader was that mannitol served as a "specific source" of carbohydrate food-supply for her organism. Since Eastcott had found that the addition of inositol to "bios II" gave an increased growth of yeast amounting to about 300 %, Reader considered that this result could similarly be explained on the grounds that inositol served as a "specific source" of carbohydrate for yeast.

This interpretation does not seem applicable to the results here described for the following reasons. Reader, using a medium containing glucose and torulin (no mannitol), obtained a reasonably good growth of her organism, under the standard conditions adopted; similarly, Eastcott, using a medium containing "bios II" without inositol obtained a 20-fold increase of yeast cells in 24 hours. On the other hand, *N. gossypii* does not give any measurable

growth even in the presence of large amounts of the baryta filtrate fraction, which corresponds to Eastcott's "bios II"; growth is of the starvation type, the surface of the medium being faintly clouded, as in the case of media containing gelatin only. It has been found possible to remove and weigh crops of mycelium amounting to 0·008 g. (dry weight, from 2 plates), and the growth of starvation type probably does not amount to more than 2–3 mg. dry weight per 2 plates. The addition of mannitol to the medium used by Reader gave a 5- or 6-fold increase of growth of *S. corallinus*; the addition of inositol to the medium in the case of *N. gossypii* resulted in a far greater increase of growth—at least 30 times. Further, the amount of mannitol used by Reader was at least 10 % of the amount of glucose in the medium, whereas in the present case active growth was produced when the inositol present was only 0·6 % of the glucose. None of the results given by Reader refers to the use of mannitol in such extreme dilution, except when torulin (corresponding to the baryta alcohol filtrate here) was also diluted. The conditions of the experiments here described seem to correspond most nearly to those in which Reader used the diluted solutions of mannitol and torulin, and in which she did not obtain "anomalous results." The evidence here is that inositol is a true growth-promoting factor, essential for healthy growth, and not merely a "specific food factor."

In this connection, an estimation was made of the amount of glucose taken from the medium during the growth of the fungus, by determining the reducing sugar present in 50 cc. of medium before and after growing the fungus thereon. It was found that a crop of the fungus, dry weight 0·28 g., had used 370 mg. of glucose during growth, the amount of inositol supplied in the medium being 8 mg. The small amount of inositol which could have been used, in comparison with the amount of glucose actually used, gave further support to the contention that inositol is more than a "specific food factor."

If the activity of inositol is to be explained on the basis of the presence of an impurity, the amount of impurity must be too small to be detected by the ordinary tests for purity, or removed by the chemical methods usually available.

Summary.

1. The statement of Farries and Bell, that *Nematospora gossypii* is unable to assimilate simple nitrogenous compounds in synthetic media, unless supplied with small amounts of an "accessory factor," is confirmed.

2. The accessory factor is shown to be present in association with crude proteins from various sources, and in extracts of lentils and etiolated lupin seedlings; cotton leaf extracts, potato, and yeast extracts show less activity.

3. The accessory factor yields two components on precipitation with barium hydroxide and alcohol; neither alone is active, the presence of both fractions being necessary.

4. The active substance in the fraction precipitated by barium hydroxide and alcohol can be replaced in the medium by small amounts of inositol.

5. The active substance present in the fraction not precipitated by barium hydroxide and alcohol is precipitated by mercuric acetate and sodium carbonate.

6. Inositol has been isolated from the baryta precipitate fraction; the fraction after removal of the inositol is inactive.

7. The activity of some relatively inactive substances (*e.g.* marmite) is greatly increased by the addition of inositol; such substances are therefore considered to contain the "baryta filtrate" fraction, and to owe their lack of activity to a deficiency of inositol.

8. Inositol is not considered to act as a "specific food factor," as is the case with mannitol in the growth of *S. corallinus* (Reader).

We wish especially to record our indebtedness to Prof. W. Brown, with whom the general research on the nutritional requirements of fungi originated, and at whose suggestion we undertook the more purely chemical investigations involved, and to Prof. A. C. Chibnall for much valuable advice and criticism.

REFERENCES.

Brown (1925). *Ann. Bot.* **39**, 373.
Eastcott (1928). *J. Physical Chem.* **32**, 1094.
Farries and Bell (1930). *Ann. Bot.* **44**, 424.
Müller (1907). *J. Chem. Soc.* **91**, 1780.
Narayanan (1930). *Biochem. J.* **24**, 6.
Reader (1928). *Biochem. J.* **22**, 434.
—— (1929). *Biochem. J.* **23**, 61.

CLXXX. THE ACCESSORY FACTOR NECESSARY FOR THE GROWTH OF *NEMATOSPORA GOSSYPII*.

II. THE RELATION OF THE ACCESSORY FACTOR TO "BIOS."

By HAROLD WILLIAM BUSTON and BIRENDA NATH PRAMANIK.

From the Biochemical Department, Imperial College of Science and Technology, London, S.W. 7.

(*Received August 28th, 1931.*)

In view of the results recorded in the preceding communication it obviously became desirable to determine whether preparations known to possess "bios" activity towards yeast could promote the growth of *N. gossypii*, and, on the other hand, whether concentrates known to show activity in respect of this fungus could serve as a source of "bios."

A study of the literature regarding "bios" shows that there is still far from general agreement as to the nature of this substance, and even as to its existence. With regard to the latter point, the work of Copping [1929] seems to summarise the present position. As the result of a survey of the nutritional needs of a very large number of yeasts, as well as other organisms, she concluded that the yeasts differ widely in their requirements; some varieties she found were able to reproduce indefinitely on synthetic media, while others, including *S. cerevisiae*, were unable to grow normally unless supplied with a factor of the "bios" type.

The chemical nature of "bios" has been and still is a matter of considerable dispute; extremely active concentrates have been prepared, without throwing any light on the identity of the substance. Claims to have separated "bios" as a chemical entity have from time to time been made, but these results have not found general acceptance. The crystalline substance isolated by Eddy *et al.* [1924], and claimed to be a pure "bios," was found to be less active than a concentrate prepared by Narayanan, and admitted to be a mixture. The concentrate of Narayanan seems to be the most active preparation yet recorded, despite its admitted impurity.

The apparent similarity of the results recorded in the preceding paper and those reported by Eastcott [1928], made it desirable to examine further the relation between the active substances necessary for the growth of *N. gossypii* and of yeast. The results indicated definitely that the two factors are not identical.

Experimental.

(1) *"Bios" activity of concentrates containing the accessory factor for* N. gossypii.

The technique adopted in the experiments on the growth of yeast was that described by Narayanan [1930]. The medium used was that of Reader [1927], which was shown by Copping [1929] to be satisfactory. The strain of yeast employed was subcultured on wort-agar slopes, and cultures of 24–30 hours' growth were used for the tests. The inoculations of the sterile Reader's medium were so made that the original yeast-count of the culture was 2500 cells per cc. Adopting the system of Narayanan, this is shown in the table as a "count" of 0·01. The cultures were incubated at 23°, and a count was made after 30 hours; a multiplication of over a hundredfold in the number of cells within this period was taken to indicate "bios" activity.

The substances tested and the results obtained are summarised in Table I. The baryta-alcohol filtrate was prepared from lentil extract after removal of the substances precipitable by phosphotungstic acid, as described in the preceding communication.

Table I.

Substance tested	Amount per 25 cc. Reader's medium (g.)	Initial count	Count after 30 hrs.
Control (medium alone)...	—	0·01	0·01
Lentil concentrate	0·001	0·01	1
,, ,, ...	0·005	0·01	2
Baryta-alcohol filtrate	0·001	0·01	0·01
,, ,, ...	0·005	0·01	0·02
Inositol	0·005	0·01	0·05
Baryta-alcohol filtrate *plus* inositol ...	0·003 + 0·005	0·01	0·13
Marmite	0·005	0·01	0·01 (?)
,, ...	0·010	0·01	1·3
,, ...	0·015	0·01	3

It appeared from these results that the lentil concentrates possessed considerable "bios" activity, and were in fact rather richer in "bios" than yeast preparations (marmite). The two main fractions prepared from the lentil concentrate, inositol and the baryta-alcohol filtrate, were both devoid of activity, and the activity was not restored on re-combining them. This seemed curious, and indicated that the "bios" substance of the lentils had been lost at some stage of the fractionation. The loss may have occurred at the phosphotungstic acid stage, since Narayanan states that "bios" is precipitated by this reagent (although the majority of other workers do not find this to be the case). Another possibility was that the baryta-alcohol filtrate fraction contained traces of inhibitory substances; there was at any rate no substance present toxic to *N. gossypii*, and the question was not pursued further. It may be noted that inositol showed no activity in promoting yeast growth, this observation agreeing with those of Narayanan. The growth produced in the

presence of inositol amounted approximately to a fivefold increase, *i.e.* to only one-twentieth of that considered as evidence of true "bios" activity. The contention of Reader and of Narayanan, that this effect is parallel with that of mannitol on the growth of *S. corallinus*, seems plausible.

(2) *Growth of* N. gossypii *in the presence of substances of known "bios" activity.*

It has been shown in the preceding paper that marmite, a product of recognised "bios" activity, is a comparatively poor source of the accessory factor for *N. gossypii*; and further, that this lack of activity is due to a deficiency of inositol. Through the kindness of Prof. J. C. Drummond, we were able to test the effect of an extremely active sample of "bios" on the growth of the fungus. This material had been prepared from yeast and was obtained as a solution containing 0·6 % of organic matter, active in promoting a vigorous growth of yeast in doses of 0·2 cc. per 2 cc. salt-glucose medium (*i.e.* 0·0012 g. solid organic matter per cc.).

When tested in the usual way for the *Nematospora* factor, the preparation was found to be entirely inactive in doses up to 0·05 g. organic matter per 50 cc. medium. That no toxic substance was present was shown by the usual tests. Since the preparation of the "bios" concentrate had involved precipitation with basic lead acetate, any inositol originally present would have been removed at this stage; to this extent, therefore, the preparation corresponded to the baryta-alcohol filtrate fraction of the lentil concentrates, and its lack of activity for *N. gossypii* was to be expected. There still remained the possibility that this "bios" preparation contained the second *Nematospora* factor, *i.e.* would give growth of the fungus when in the presence of inositol. Unfortunately, lack of material made it impossible to test this point. Recent work on other lines has given definite indications that this is not the case, and that although concentrates of the accessory factors for *N. gossypii* may show "bios" activity, the factor responsible is quite distinct from those promoting growth of the fungus.

SUMMARY.

The growth-promoting accessory necessary for *N. gossypii* is found in association with "bios" among natural products, but the two substances are not identical. Inositol is devoid of true "bios" activity, but is essential for the growth of *Nematospora*.

REFERENCES.

Copping (1929). *Biochem. J.* **23**, 1050.
Eastcott (1928). *J. Phys. Chem.* **32**, 1094.
Eddy, Kerr and Williams (1924). *J. Amer. Chem. Soc.* **46**, 2846.
Narayanan (1930). *Biochem. J.* **24**, 6.
Reader (1927). *Biochem. J.* **21**, 904.

CLXXXI. THE RÔLE OF CERTAIN DIETARY FACTORS IN THE FORMATION OF ERYTHROCYTES.

By BIRES CHANDRA GUHA[1]
and LESLIE WILLIAM MAPSON.

From the Biochemical Laboratory, Cambridge.

(*Received August 17th, 1931.*)

Our knowledge about the precursors and catalysts involved in the production of red blood cells is very meagre. The most important advances in this field during recent years have been the recognition of copper as an important element concerned in the formation of haemoglobin [Hart *et al.* 1928] and of the principles present in liver, which are curative of pernicious anaemia [Minot and Murphy, 1926] which are now considered to be a peptide composed of β-hydroxyglutamic acid and *l*-γ-hydroxyproline [Dakin, West and Howe, 1931] and the more recently discovered tribasic acid of the composition $C_{11}H_{15}O_7N$, H_2O which gives reactions of the pyrrole group [Dakin and West, 1931]. The observation that a commercial liver extract (Eli Lilly and Co., No. 343) was a very potent source of vitamin B_2 and cured symptoms of depilation in rats [Guha, 1931, 1, 2] and was at the same time a product highly effective in pernicious anaemia raised the question of a possible relationship between the two factors concerned. While the possibility of a chemical identity was excluded on several grounds [Guha, 1931, 2, 3], it was considered of interest to investigate whether vitamin B_2 had a similar haematopoietic effect. The recent observations of Bliss [1930] and of Bliss and Thomason [1931] on the relation of pellagra to the blood picture, lent further point to this question. The rôle of vitamin B_2 in the economy of the organism is at present not understood. Hassan and Drummond [1927] observed that a high protein diet required a larger allowance of the relatively heat-stable component of marmite, which was presumably vitamin B_2, for the good growth of rats. Kon [1929] observed an increase in the urinary C/N ratio in vitamin B_2 deficiency. But no conclusive evidence was obtained by Kon [1931] to show that vitamin B_2 has a specific influence on nitrogen metabolism. Leader's work [Leader, 1930], on the other hand, tends to show that sugar in the diet has a predisposing effect towards the production of experimental pellagra. In fact, although a considerable number of attempts have been made to correlate one or other of the vitamins with processes of general metabolism, such studies cannot be said to have yielded so far any significant results. It

[1] Travelling Fellow of the University of Calcutta.

was desirable, therefore, to attempt to explore the mode of action of vitamin B_2 in a different direction.

While this work was in progress, Sure, Kik and Smith [1931] published a note, in which they reported a diminution in haemoglobin concentration and in the red blood cell count in vitamin B_2 deficiency, when it was accompanied by skin lesions. This type of anaemia was not observed in deficiency of vitamins A, D or E and it was considered that the secondary anaemias sometimes observed in keratomalacia in man were probably due to secondary disorders and not specifically to vitamin A deficiency [Sure, Kik and Walker, 1929, 2; Sure, Kik and Walker, 1931; Sure and Kik, 1931]. Sure, Kik and Walker [1929, 1] had, however, formerly observed fluctuations in the concentration of erythrocytes and haemoglobin in rats on diets deficient in the vitamin B complex. These results appear to indicate a certain degree of specificity in the diminution of the erythrocyte concentration in vitamin B_2 deficiency.

The effect of minute amounts of copper in nutritional anaemia has been shown by Hart *et al.* [1928]. While, however, these authors found no evidence for the existence of an organic substance necessary for haemoglobin regeneration in rats on a whole milk diet, Drabkin and Miller [1931] have recently reported the efficacy of certain amino-acids, glutamic acid and arginine in particular, in cases of nutritional anaemia in the rat. In view of these statements and of the suggestion of Bliss [1930] that pellagra might be due to simple iron deficiency, the combined effect of copper, iron and glutamic acid on vitamin B_2-deficient rats has been studied. The effect of vitamin B_1 deficiency has also been studied for purposes of comparison.

It has been recorded before [Guha, 1931, 2] that an aqueous extract of liver suffers a great loss in vitamin B_2 on being autoclaved in an alkaline medium. As one of us (L. W. M.) has found, in course of an investigation shortly to be published on the rôle of liver in normal nutrition, that the feeding of fresh liver produces a marked increase in the red cell count of normal rats, it was of interest to enquire whether the feeding of an aqueous liver extract, in which vitamin B_2 was destroyed by autoclaving, was capable of raising the erythrocyte count of vitamin B_2-deficient animals. The results obtained indicate the existence of a heat-stable factor in liver, which has a most pronounced effect on the formation of red blood cells. That it is probably different from the factor responsible for the cure of pernicious anaemia appears from considerations, which will be discussed later.

The present work, which was begun to investigate the relation, if any, between vitamin B_2 and the erythrocyte count, has thus been extended to cover the following points.

1. The effect of vitamin B_2 deficiency, followed by administration of vitamin B_2, on the red cell count.

2. The influence of copper, iron and glutamic acid, if any, on the erythrocyte count in vitamin B_2 deficiency.

3. The question of the iron intake in vitamin B_2 deficiency.
4. Other dietary factors concerned in the production of erythrocytes.
5. The effect of vitamin B_1 deficiency on the red cell count.

Experimental.

Both albino and piebald rats, raised in this laboratory, were used. Blood for red cell counts was taken after clipping the end of the tail, and counts were taken with a Bürker haemocytometer. Haldane's method was used for estimations of haemoglobin, but as enough blood was not always obtainable for accurate estimations, these were carried out only in a small number of cases. Whether the haemoglobin concentration goes hand in hand with the red cell count or not, will, therefore, have to be settled by further work, which is in progress.

The following basal diets were fed in these experiments.

Diet 16	%
Rice starch	65
Palm kernel oil	10
"Light white casein"	21
Salt mixture (Osborne and Mendel)	4

Diet 16 (*a*)	%
Rice starch	40
Cane sugar	17
"Light white casein"	23
Palm kernel oil	15
Salt mixture (Osborne and Mendel)	5

Diet 16 (*b*) was made up by incorporating 12 g. dried yeast per 100 g. of diet 16.
Diet 16 (*c*) was made up by incorporating 10 g. dried yeast per 100 g. of diet 16 (*a*).

All the animals received 2 drops of cod-liver oil daily. All other supplements were also fed separately from the basal diet unless otherwise stated. The vitamin B_1 preparation was obtained by the extraction of brewer's top yeast with boiling water and precipitation with neutral lead acetate, followed by adsorption with and elution from fuller's earth [Guha, 1931, 4]. Such a preparation provides an excellent source of vitamin B_1, practically free from vitamin B_2 [Guha, 1931, 2]. The vitamin B_2 preparation was obtained in the usual way by autoclaving marmite with baryta at p_H 9 for $1\frac{1}{2}$ hours at 18 lbs. pressure. Such a preparation, while it is a rich source of vitamin B_2, contains negligible quantities of vitamin B_1 [Guha, 1931, 2, 4]. The aqueous liver extract, free from vitamin B_2 was obtained by a modification of the method described before [Guha, 1931, 2]. Fresh ox-liver (100 g.) was finely minced, suspended in distilled water (300 cc.), brought to p_H 5 with dilute HCl, and boiled up for 5 minutes. The filtrate was brought to p_H 9 with dilute NaOH and autoclaved for $1\frac{1}{2}$–3 hours at 18 lbs. pressure. The resulting preparation (3 cc. = 1 g. of fresh liver) was almost entirely devoid of vitamin B_2 (Fig. 1).

The copper sulphate, ferric chloride and glutamic acid were administered in daily doses of 0·1 cc., 0·5 cc. and 1 cc. of aqueous solutions containing respectively 0·05 mg. copper sulphate, 0·5 mg. ferric chloride and 50 mg. glutamic acid.

The figures for the red blood cell count are given throughout in millions per mm.^{3} The leucocyte counts of a few vitamin B_2-deficient rats were compared

with those of normal animals but no marked differences were observed [see also Sure, Kik and Smith, 1931].

A. In these experiments animals, which had been previously undergoing partial vitamin B_2 deficiency, were subjected to relatively short periods of complete vitamin B_2 deficiency (3–4 weeks) and then their red cell counts

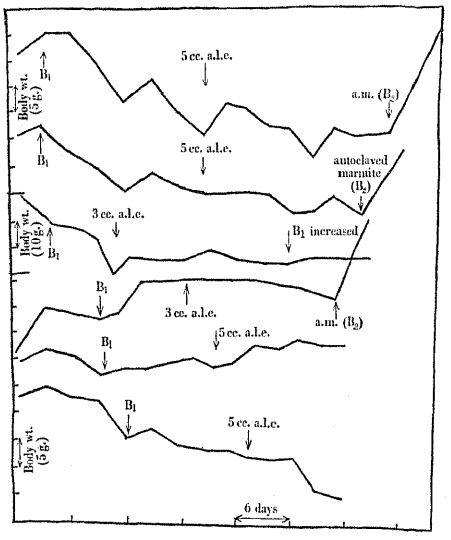


Fig. 1. Curves illustrating the ineffectiveness of autoclaved liver extract to supply vitamin B_2.
a.l.e. = autoclaved liver extract. a.m. = autoclaved marmite.

were taken. They were receiving throughout 1 cc. of the vitamin B_1 preparation, which was found from separate experiments to be ample. It was considered that the physiological rôle of vitamin B_2 should be more apparent under these circumstances than under drastic deprivation of vitamin B_2, as in this latter case secondary complications might mask the primary effect. The results are given in Table I.

Table I.

Rat No.	Colour	Sex	Age (weeks)	Period of complete vitamin B_2 deficiency (days)	Body wt. at the time of taking R.B.C. (g.)	R.B.C.	Average R.B.C.
106	Black and white	♂	25	25	129	8·0	
107	,,	♀	25	30	117	7·7	
108	,,	♀	25	30	129	7·8	
109	,,	♀	25	30	125	7·0	7·78
155	Albino	♂	20	30	105	7·9	
156	,,	♂	20	25	92	8·3	
157	,,	♂	20	30	112	7·5	
160	,,	♂	20	25	109	8·0	

The figures for normal rats, subsisting on milk, brown bread and whole wheat, of approximately the same age were 9·2, 9·2, 9·2 and 9·4, giving an

average of 9·25 millions/mm.^{3} The reduction in the red cell count in vitamin B_2 deficiency was thus relatively small but distinct, although symptoms of depilation or dermatitis had not yet supervened in these animals.

B. In these experiments, the effect of copper, iron, glutamic acid and autoclaved marmite on vitamin B_2-deficient animals was investigated. The vitamin B_1 preparation was fed in 1 cc. daily doses to all the rats. Copper sulphate, ferric chloride and glutamic acid, whenever fed, were administered in daily doses of 0·05 mg., 0·5 mg. and 50 mg. respectively. These three substances gave negative results. The effect of autoclaved marmite, in a daily dose equivalent to 0·5 g. of marmite, is shown in Fig. 2.

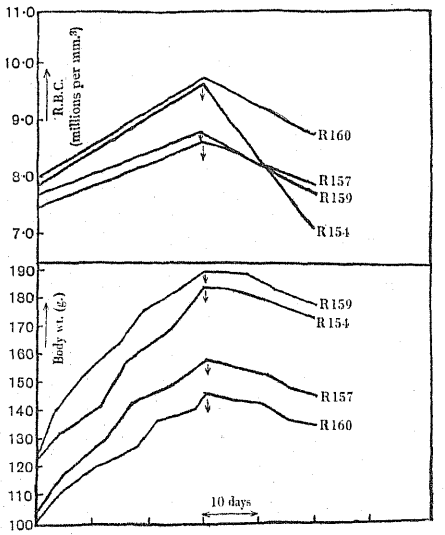


Fig. 2. Fuller's earth extract (B_1) *plus* autoclaved marmite (B_2) from beginning of exp.; ↓ autoclaved marmite withdrawn.

These results show that the diminution of the red cell count occurring in vitamin B_2 deficiency can be corrected by alkaline autoclaved marmite but not by copper, glutamic acid and additional iron.

In order to test the view of Bliss [1930], who considers vitamin B_2 deficiency as simply iron deficiency, the food intake records of the above animals were kept for 10 days, although the fact that rats Nos. 106, 155 and 156, which were receiving extra iron separately from the basal diet, showed no gain in weight, would by itself indicate that iron cannot replace vitamin B_2 for the growth of rats. The figures for the food consumption are given in Table II.

While these figures show that there is a distinct reduction in food intake in vitamin B_2 deficiency, it appears that 8·2 g. of the basal diet still carry enough iron to cover the iron requirements of the animals, as the salt mixture contained 3·2 % ferric citrate. The ineffectiveness of additional iron in the absence of vitamin B_2 also appears to confirm this view. In order to test this point further, rats Nos. 157 and 160, which were receiving autoclaved marmite,

were given restricted amounts of food (7 g. daily) for 10 days before the final erythrocyte count was taken, but in spite of this artificially lowered food consumption, they gave normal figures, which indicates that even 7 g. of the food provided a reasonable amount of iron. The vitamin B_2-deficient rats were normally consuming 8·2 g. of food daily on the average but still gave lower red cell counts.

Table II.

Rat No.	Daily food intakes for 10 days (g.)										Average daily food intake (g.)	
107	8	8	9	8	7	7	10	9	8	12	8·6	
108	6	6	9	7	7	7	10	6	10	9	7·7	8·2
109	10	8	8	8	8	9	8	8	7	9	8·3	
106	11	7	5	4	6	9	10	8	6	7	7·3	
155	4	5	6	6	8	7	8	7	6	6	6·3	6·3
156	3	5	3	6	5	6	6	7	6	6	5·3	
154	10	9	10	11	11	12	14	14	11	13	11·4	
159	7	10	11	13	11	10	12	12	12	13	11·1	10·9
157	11	10	9	11	11	11	11	12	9	11	10·6	
160	6	10	10	10	12	11	14	11	11	11	10·6	

C. The supplements administered to the animals in the above experiments were then interchanged in some cases and three of the animals were given a daily dose of 2 cc. of the autoclaved liver extract each—a preparation practically free from vitamin B_2 (Figs. 3 and 4). The striking effect of this

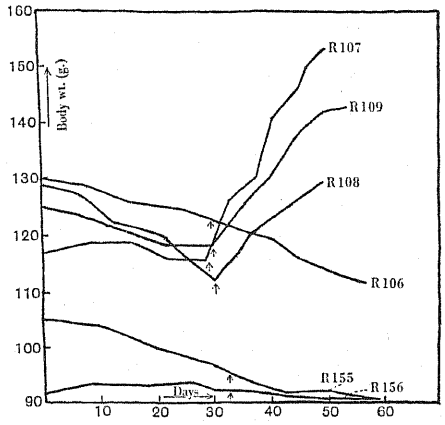


Fig. 3.

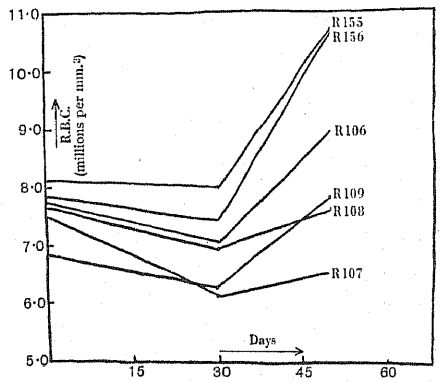


Fig. 4.

Fig. 3. R 107, R 108 and R 109, fed diet 16 + B_1, from beginning of exp.; ↑ 1 cc. autoclaved marmite in addition. R 106, R 155 and R 156, fed diet 16 + B_1 + Cu + Fe + glutamic acid from beginning of exp.; ↑ 2 cc. autoclaved liver extract in addition.

Fig. 4. Figures for red cell counts, corresponding to growth-curves shown in Fig. 3.

extract on the blood picture, in spite of the arrest of growth due to the absence of vitamin B_2 from the diet, is evident from the figures given in Table III.

It will be observed that the administration and withdrawal of autoclaved marmite were followed by a perceptible rise and fall in the red cell count. It is, however, important to notice that autoclaved liver extract, which does

not contain vitamin B_2, produces a much more marked effect than autoclaved marmite.

Table III.

Rat No.	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	Initial R.B.C.	Average initial R.B.C.	Final R.B.C.	Average final R.B.C.
107	$16+B_1+B_2$	20	119	152	6·3		6·6	
108	,,	20	111	128	7·1	6·6	7·8	7·5
109	,,	20	118	141	6·4		8·0	
106	$16+B_1$+autoclaved liver extract	20	122	113	7·2		9·1	
155	,,	20	92	92	7·6	7·7	10·9	10·3
156	,,	20	98	92	8·2		10·8	
154	$16+B_1$	20	191	181	9·8		7·1	
159	,,	20	196	186	8·8	9·3	7·7	7·6
157	,,	20	166	153	8·7		7·9	
160	,,	20	154	143	9·8		7·8	

D. Somewhat similar experiments were carried out with a group of young animals in order to compare the effects produced by (*a*) autoclaved liver extract alone, (*b*) autoclaved liver extract *plus* autoclaved marmite, and

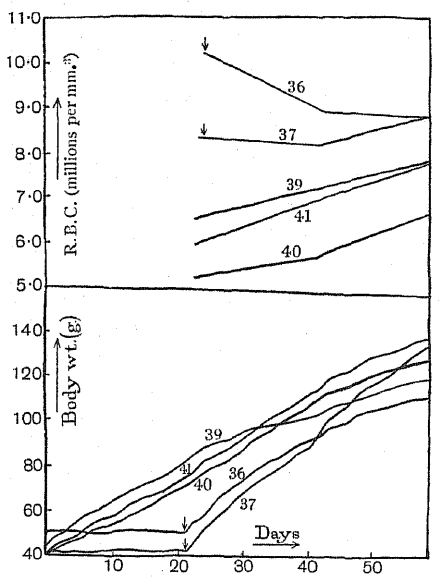


Fig. 5. Rats No. 39, 40 and 41 fed on diet 16 (*b*) throughout. Rats No. 36 and 37 fed on diet 16 + B_1 + autoclaved liver extract (3 cc.); ↓ 1 cc. autoclaved marmite in addition.

(*c*) copper, iron and glutamic acid together with a diet containing 12 % yeast (diet 16 (*c*)). The autoclaved liver extract and autoclaved marmite were fed in 3 cc. and 1 cc. daily doses respectively. In these experiments the effect of feeding the same or different supplements for different periods on the same rat was studied, the animals thus serving as their own controls. The results are given in Fig. 5.

These results indicate that the effect of 12 % yeast in the diet, supple-

mented by copper, glutamic acid and extra iron, on the red cell count, is not at all comparable with the pronounced effect of autoclaved liver extract. It also appears that the administration of autoclaved marmite in addition to autoclaved liver extract does not further stimulate the formation of erythrocytes (see also Section E). If anything it appears to have an inhibitory effect. This is in contrast to the stimulating effect of autoclaved marmite, when given by itself to rats, which had been previously undergoing vitamin B_2 deficiency (see Tables II and IV). The significance of these results is discussed later.

E. The results of experiments, similar to those described in the preceding section, on another group of rats, approximately 8 weeks old, are given in Table IV. The autoclaved liver extract and autoclaved marmite were fed in

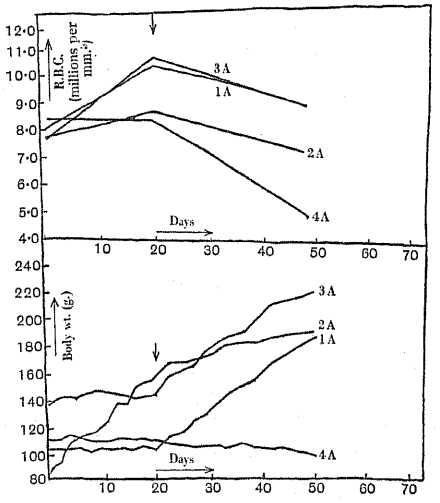


Fig. 6. 1 A and 3 A fed on diet 16 + B_1 + autoclaved liver extract; ↓ 1 cc. autoclaved marmite in addition, 2 A fed on diet 16 + B_1 + autoclaved marmite throughout, 4 A fed on diet 16 + B_1 + Cu + Fe + glutamic acid; ↓ 16 + B_1 + 0·5 cc. alkaline haematin.

3 cc. and 1 cc. daily doses respectively. In this set of experiments the effect of different supplements on the same rat was studied. In previous communications [Guha, 1931, 2, 5] it was stated that haemin, administered in a fine aqueous suspension, was unable either to restore the growth or cure the symptoms of depilation of vitamin B_2-deficient rats. As much of the haemin fed was excreted and as Kollath's experiments [1929, 1930] were carried out with alkaline haematin, 0·5 cc. of a solution of haemin in dilute sodium hydroxide containing 2·5 mg. of haemin was fed daily to one of the rats of this group with entirely negative results. As this was not taken readily it had to be incorporated in the diet. The daily administration of this alkaline solution appeared to have a deleterious effect (Fig. 6).

These results again demonstrate the quite noticeable effect of autoclaved marmite on the red cell count, while the effect of autoclaved liver extract is

very marked. The results with copper, glutamic acid and alkaline haematin are negative. The curious fact is again noticed that on feeding autoclaved marmite in addition to autoclaved liver extract, the red cell count has a tendency to fall (rats 1 A and 3 A). This confirms the experiments described in the preceding section.

Table IV.

Rat No.	Colour	Sex	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	Initial R.B.C.	Final R.B.C.	Final haemoglobin concentration (g. in 100 cc. blood)
1 A	Albino	♂	16 + B_1	25	—	107	—	8·0	—
,,	,,	,,	16 + B_1 + autocl. liver ext.	20	107	108	8·0	10·3	—
,,	,,	,,	16 + B_1 + autocl. liver ext. + autocl. marmite	27	108	183	10·3	8·9	14·6
2 A	,,	,,	16 + B_1	25	—	82	—	7·7	—
,,	,,	,,	16 + B_1 + autocl. marmite	20	82	157	7·7	8·6	—
,,	,,	,,	,,	27	157	187	8·6	7·0	14·0
3 A	,,	,,	16 + B_1	25	—	136	—	7·6	—
,,	,,	,,	16 + B_1 + autocl. liver ext.	20	136	146	7·6	10·6	—
,,	,,	,,	16 + B_1 + autocl. liver ext. + autocl. marmite	27	146	219	10·6	8·9	15·4
4 A	Black and white	,,	16 + B_1	25	—	113	—	8·3	—
,,	,,	,,	16 + B_1 + $CuSO_4$ + $FeCl_3$ + glutamic acid	20	113	104	8·3	8·4	—
,,	,,	,,	16 + B_1 + alk. haematin	27	104	98	8·4	4·6	10·6*

* On the day on which the last estimations were made this animal was extremely miserable and nearly dying.

F. Seven young black and white rats of the same litter, which were split up into three groups and were fed on basal diets (diets 16 (*a*) and 16 (*c*)), slightly different from the basal diets fed in the foregoing experiments, gave the following figures for the erythrocyte and haemoglobin concentrations (Table V). The autoclaved liver extract was fed in 2 cc. daily doses.

Table V.

Rat No.	Sex	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	R.B.C.	Average R.B.C.	Hb (g. per 100 cc. blood)	Average Hb (g. per 100 cc. blood)
65	♀	16 (*c*)	30	67	130	7·3	7·1	12·5	13·0
66	,,	,,	30	57	117	6·9		13·5	
67	,,	16 (*a*) + B_1	30	70	89	6·4	6·35	11·2	11·3
68	,,	,,	30	80	88	6·3		11·4	
69	,,	16 (*a*) + B_1 + autocl. liver ext.	30	66	84	9·9	9·17	13·5	13·6
70	,,	,,	30	70	84	9·0		13·5	
71	,,	,,	30	60	79	8·6		13·8	

These results demonstrate that the presence of yeast in the diet (diet 16 (*c*)) gives somewhat higher figures for the red cell and haemoglobin concentrations

than when the basal diet (16 (*a*)) is supplemented only by vitamin B_1. But the addition of autoclaved liver extract to the diet gives a much higher figure for the red cell count than can be obtained with yeast, while the effect on the haemoglobin concentration is not so marked.

G. It was desirable to get a few figures for the red cell count in vitamin B_1 deficiency. Animals of approximately the same age were given synthetic diets with and without vitamin B_1 and the red cell concentrations of their peripheral blood estimated in the usual way (Table VI).

Table VI.

Rat No.	Colour	Sex	Nature of diet	Period of feeding on diet (days)	Body wt. at the time of taking R.B.C.	R.B.C.	Average R.B.C.
25	Albino	♀♀♀	$-B_1$	30	71	10·5	
27	,,		$-B_1$	30	81	10·0	10·53
28	,,		$-B_1$	30	67	11·1	
21	,,	♀♀♀	$+B_1$	30	115	9·8	
22	,,		$+B_1$	30	130	10·4	10·33
23	,,		$+B_1$	30	104	10·8	

While the number of these experiments is inadequate to justify definite conclusions, it appears that in vitamin B_1 deficiency, in contradistinction to vitamin B_2 deficiency, there is no reduction in the red cell count. Sure, Kik and Walker [1929,1] observed a slight rise in the red cell count in vitamin B_1 deficiency, which was accompanied by anhydraemia.

Discussion.

Whipple and his co-worker [1925], working chiefly with dogs, have shown in a series of important papers that certain food materials possess a very marked blood regenerative power in experimental secondary anaemia. The specific effect of copper in nutritional anaemia, observed by Hart *et al.* [1928], has been confirmed by Titus, Cave and Hughes [1928], who have also observed a specific effect of manganese. Beard and Myers [1929] have added nickel, cobalt and germanium to this list. It is necessary to stress the point that the factors, effective in one type of anaemia, may not be effective in a different type. Thus Robscheit-Robbins and Whipple [1929] found that Eli Lilly's commercial liver extract, No. 343, which is highly effective in cases of pernicious anaemia, was not nearly so effective in severe secondary anaemia in dogs. It is, therefore, not possible to say how far the factors with which we have been dealing in the present paper come into the picture of clinical and experimental anaemias.

The principle present in the aqueous extract of fresh ox-liver, which gives rise to supernormal concentration of erythrocytes in blood, even when the rats are in poor condition owing to vitamin B_2 deficiency, is apparently heat-stable. This response cannot be due to iron and copper, present in the liver extract, as these elements have been shown in this paper to be quite ineffective

in producing this unusual response when fed by themselves. The failure of glutamic acid, which, according to Drabkin and Miller [1931], is effective in nutritional anaemia in the rat, indicates that the principle in liver is of a different nature. Though we have not yet obtained conclusive evidence that this substance is different from the factors concerned in the cure of pernicious anaemia, an identity between the two appears to be improbable. Thus, as has been pointed out above, the factors curative of pernicious anaemia have been stated to be of relatively slight effect in cases of secondary anaemia, and it is, therefore, improbable that the factor in fresh liver, described in this paper, which exhibits its effect apparently even under normal conditions, is identical with them. Preliminary experiments with the E.L. liver extract No. 343 indicate that it is not nearly so effective under our conditions of experiment as autoclaved liver extract. Moreover, it would appear from the paper of Cohn, Minot, Alles and Salter [1928] that the factor curative of pernicious anaemia described there is not particularly heat-stable, likewise the acid described by Dakin and West [1931] appears to be unstable under the conditions in which the autoclaved liver extract described in this paper is made. Further work is being done in order to settle this question.

The present work indicates that there is a diminution in the red cell concentration of blood in vitamin B_2 deficiency, which can be restored to normal by feeding autoclaved marmite, though, unlike the autoclaved liver extract, this was not found to push the erythrocyte concentration beyond the normal level. While the evidence is suggestive and prompts further investigation, it cannot yet be definitely stated that the factor concerned, which is present in autoclaved marmite, is vitamin B_2. Doubt is cast on this point chiefly by the consideration that the diminished red cell count occurring in vitamin B_2 deficiency can be increased above the normal by feeding autoclaved liver extract, which is practically free from vitamin B_2. While this experiment does not by any means show that vitamin B_2 has no haematopoietic effect, it is clear that it is possible to raise the red cell count in the almost complete absence of vitamin B_2. Whether the factor present in autoclaved marmite, which restores the red cell count to normal, is vitamin B_2 or is identical with the liver principle can be settled by obtaining a preparation of vitamin B_2, which is free from the liver factor. We have not yet succeeded in this objective, though we have been able to obtain a liver preparation, which is free from vitamin B_2.

The question of the aetiology of pellagra has been discussed elsewhere [Guha, 1931, 5]. It is obvious from the experiments described in this paper, in which food intake records were taken and additional iron was fed to some of the animals, that the arrest of growth due to vitamin B_2 deficiency cannot be due simply to iron deficiency. The reduction in the red cell count, occurring in vitamin B_2 deficiency can be corrected by autoclaved marmite but not by copper, glutamic acid or additional iron. Though we are not yet certain that this principle in autoclaved marmite is vitamin B_2, there appears to be enough

suggestive evidence to warrant the possibility that vitamin B_2 may be concerned in some phase of the mobilisation of ingested iron. Bliss and Thomason [1931] point out that, according to Elvehjem and Peterson [1927], hair is the richest source of iron in the body, and it does not appear impossible that the depilation, occurring in vitamin B_2 deficiency, might be at least partly due to a failure in this phase of iron metabolism.

Certain figures quoted by us indicate that there might be a question of balance between different factors concerned in the formation of erythrocytes. Thus the rate of erythrocyte formation due to the feeding of autoclaved liver extract appeared to be inhibited by the administration of autoclaved marmite. This inhibition was, of course, associated with increased growth produced by autoclaved marmite. Funk and Lejwa [1931], in a recent paper to which our attention was drawn after this work had been completed, mention that rapid growth induces a decrease in the number of red cells, and they consider that there are factors present in foodstuffs which stimulate, and others which inhibit, blood regeneration.

It may be interesting to state that some of the vitamin B_2-deficient animals, which were receiving vitamin B_1 and autoclaved liver extract, lost fur more readily than those receiving vitamin B_1 only, suggesting that the liver principle might hasten depilation by mobilising most of the available iron for the generation of blood, when the food intake and, therefore, the iron intake is limited.

It appears from the few measurements of haemoglobin, that we have made, that the influence of autoclaved liver extract on the concentration of red cells is relatively greater than on the concentration of haemoglobin. This might indicate a more specific effect of the liver principle on the formation of the stroma of erythrocytes.

Summary.

1. Rats under vitamin B_2 deficiency show a diminution in the red cell count, which can be restored to normal by autoclaved marmite but not by copper, glutamic acid, extra iron or alkaline haematin.

2. The arrest of growth in vitamin B_2 deficiency cannot be due to simple iron deficiency, as is shown by records of food consumption. Alkaline haematin cannot replace vitamin B_2 in the diet.

3. Vitamin B_1 deficiency does not result in a reduction of the red cell count.

4. The presence of a heat-stable factor in ox-liver is demonstrated, which exerts a powerful influence on the formation of erythrocytes.

5. This factor can be extracted with boiling water and can be freed from vitamin B_2 by autoclaving in an alkaline medium. Even when growth is arrested and the animals are in poor condition owing to vitamin B_2 deficiency, the administration of this autoclaved liver extract induces a supernormal

erythrocyte count. This effect cannot be produced by iron, copper, glutamic acid and yeast.

6. The significance of these results is discussed.

We wish to express our gratitude to Sir F. G. Hopkins for his kind interest and advice. We owe much to the skilful assistance of Miss R. Leader.

REFERENCES.

Beard and Myers (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 510.
Bliss (1930). *Science*, **72**, 578.
—— and Thomason (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 636.
Cohn, Minot, Alles and Salter (1928). *J. Biol. Chem.* **77**, 325.
Dakin, West and Howe (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
—— and West (1931). *J. Biol. Chem.* **92**, 117.
Drabkin and Miller (1931). *J. Biol. Chem.* **90**, 531.
Elvehjem and Peterson (1927). *J. Biol. Chem.* **74**, 433.
Funk and Lejwa (1931). *Bruxelles-Méd.* **11**, 331.
Guha (1931, 1). *Lancet* i, 864.
—— (1931, 2). *Biochem. J.* **25**, 945.
—— (1931, 3). *Nature*, 594.
—— (1931, 4). *Biochem. J.* **25**, 931.
—— (1931, 5). *Brit. Med. J.* (ii), 53.
Hart, Steenbock, Waddell and Elvehjem (1928). *J. Biol. Chem.* **77**, 797.
Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
Kollath (1929). *Arch. exp. Path. Pharm.* **142**, 86.
—— (1930). *Arch. exp. Path. Pharm.* **150**, 236.
Kon (1929). *J. Nutrition*, **1**, 46.
—— (1931). *Biochem. J.* **25**, 482.
Leader (1930). *Biochem. J.* **24**, 1172.
Minot and Murphy (1926). *J. Amer. Med. Assoc.* **87**, 470.
Robscheit-Robbins and Whipple (1929). *J. Exp. Med.* **49**, 215.
Sure and Kik (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 496.
—— —— and Smith (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 498.
—— —— and Walker (1929, 1). *J. Biol. Chem.* **83**, 387.
—— —— —— (1929, 2). *J. Biol. Chem.* **83**, 401.
—— —— —— (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 495.
Titus, Cave and Hughes (1928). *J. Biol. Chem.* **80**, 565.
Whipple and Robscheit-Robbins (1925). *Amer. J. Physiol.* **72**, 395.

CLXXXII. A SURVEY OF ANTHOCYANINS. I.

By GERTRUDE MAUD ROBINSON and ROBERT ROBINSON.

From the Dyson Perrins Laboratory, Oxford.

(*Received August 18th, 1931.*)

Although the details of the constitution and properties of the anthocyanins can only become known as the result of their isolation in substance and the study of the pure individual pigments following the lines of the pioneering researches of Willstätter and his collaborators [1913–16], yet it is also true that much can now be gleaned from an examination of the colouring matters in solution and there are many obvious advantages attaching to this procedure.

For example, the formation and occurrence of anthocyanins can be instantly recognised by inspection and the development of a method of analysis is a necessary step towards the utilisation of this property in making attempts to solve the problems of the origins and functions of these pigments. Moreover, phylogenetic investigations are often facilitated by a knowledge of the anthocyanins of the varieties, and more rapid methods than those demanding the collection of kilograms of the dried petals are urgently required.

Finally a preliminary survey should afford evidence of the existence of interesting new types of anthocyanins, and thus pave the way for extensions of the large scale investigations.

In all cases we have employed cold 1 % aqueous hydrochloric acid in order to extract the coloured materials and the best results were obtained by using fresh flowers and by examining the solutions as soon as possible following their preparation.

Colour of flowers and of the extracts—co-pigments.

The colours of the flowers or fruits themselves do not afford trustworthy indications, but whilst orange-scarlet shades indicate the occurrence of a pelargonidin derivative no blue flower-pigments are derived from pelargonidin. On the other hand not very dissimilar bluish-red *Phlox*, crimson *Dianthus* and crimson-scarlet *Linum* contain anthocyanins derived respectively from pelargonidin, cyanidin and delphinidin.

More reliance can be placed on the colour of the acid extracts provided these are observed at the boiling-point of the solution. Under these conditions the order from orange-red to blue-red is that of anthocyanins based on pelargonidin, peonidin, cyanidin, malvidin and delphinidin.

The necessity for this precaution arises from the existence in the solutions of substances (co-pigments) which intensify and modify the colour. Willstätter

and Zollinger [1916] observed that the addition of tannin to a solution of oenin chloride in dilute hydrochloric acid intensified the colour and produced a change in tone giving a much bluer red. These authors also noticed that gallic acid had a similar but weaker effect and that the ferric reactions and alkali-colour reactions were also modified; finally they commented on the fact that the effect is specific for oenin and is not observed to a comparable extent in the case of cyanin solutions.

This is true for gallotannin (although malvin is as sensitive as oenin) but co-pigments exist for all the types of anthocyanins, although they have not yet been identified. Some preliminary experiments in this field are described in the experimental section, and we note here in illustration of specificity that the glucoside of 2-hydroxyxanthone is a powerful co-pigment for cyanin but not for mecocyanin, and that the isomeric glucoside of 4-hydroxyxanthone is far less active.

The phenomenon has little or nothing to do with salt formation and occurs in the presence of a large excess of mineral acid; it is evidently the result of the formation of weak additive complexes which are dissociated at an elevated temperature or by the action of solvents.

We have reason to believe that the formation of these complexes occurs in the flowers themselves and plays a most important part in producing variations of colour. Our interest in this aspect of the subject arose from an examination of the fuchsia. The violet inner corolla gives a bluer red acid extract than that obtained from the outer bluish-red petals, and this was at first regarded as a proof that the anthocyanins are different in the two cases. We still believe that this is part of the truth, but it was noticed that the blue-red solution from the violet petals became much redder on washing with amyl alcohol. Addition of light petroleum to the separated amyl alcohol and extraction of the mixture with 0·5 % hydrochloric acid furnished an extract which, mixed with the washed anthocyanin solution, reproduced the original blue-red solution. In this case the substance in the amyl alcohol was tannin, identified as derived gallic acid. Subsequently the phenomenon was found to be very general and some of the effects are surprisingly great and cannot yet be imitated with synthetic compounds.

Willstätter and Mallison [1915] found that the rose-coloured flowers of *Pelargonium peltatum* contained pelargonin, the colouring matter of the scarlet pelargonium, and concluded that the cell-sap of the bluer red variety must be the more alkaline.

The aqueous acid extract of the rose-coloured petals is, however, also bluish-red and acquires the colour of a pure pelargonin solution only on heating; on cooling the blue shade returns. The bluish-red phlox is another striking instance of the occurrence of a pelargonin-co-pigment complex.

It seems that great changes in the colour of varieties in a species are not brought about by changes in the p_H of the cell-sap, but rather by changes in the nature of the anthocyanin, including in this term the formation of complexes

with organic substances and possibly with metals such as iron. Haas and Hill [1929] have expressed a similar view.

An apparent exception is *Primula sinensis*, in magenta and blue varieties of which Miss R. Scott-Moncrieff has found the same anthocyanins. Here, we think that one of the genetic factors concerned is a quantitative one; the magenta flowers are much the more intensely coloured and the modifying substances present in low concentrations are unable to deal with the whole amount. In the pale-coloured rather violet-shaded blue flowers the pigment remains the same, but it is all modified according to the present hypothesis. We wish to emphasise that the evidence for the theory is that changes in colour are so often found associated with a change in the anthocyanin and that we speak only of closely related varieties or species. It is agreed that changes of p_H in the cell-sap occur at different stages in the life of the plant and also in representatives of distinct genera; for example, Willstätter and Everest [1913] made it very probable that the colouring matter of the blue cornflower is the potassium salt of cyanin, whereas the rose and dahlia contain the pigment in the form of an oxonium salt with an organic acid. It is obvious also that a genetic factor for flower colour may be concerned only with the development of a co-pigment, the anthocyanin remaining unchanged in composition and concentration.

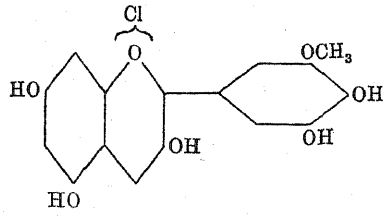
Methods of examination of the extracts and scope of the results.

The details are given in the experimental section, but in general we have observed the colour reactions with alkalis and ferric chloride and made considerable use of distributions between immiscible solvents.

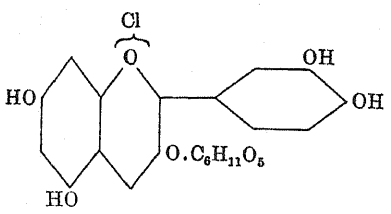
The value of this method was indicated by Schudel [1918] whose dissertation was, we understand, the result of work in Prof. Willstätter's laboratory. Willstätter and Schudel developed a technique for the separation of anthocyanidins, monoglycosidic[1] and diglycosidic anthocyanins and employed a similar process in the difficult isolation of betanin from the beet. We have made use of their observations in order to devise methods of purification of the anthocyanidins and anthocyanins and to assist in characterising the pigments.

Apart from the betanidin group and the yellow anthocyanin observed by Willstätter in the flowers of *Papaver alpinum*, it has not been found necessary to assume the existence of new anthocyanidins—we have not proved that such do not exist in the material examined, but we can accommodate our results without them. Nevertheless we are now of the opinion that petunidin chloride [Willstätter and Burdick, 1916] is essentially 3′-*O*-methyldelphinidin chloride (I) identical with the salt synthesised by Bradley, Robinson and Schwarzenbach [1930].

[1] The term "glycosidic" is employed when the nature of the sugar is unknown; "glucosidic" being used to denote a derivative of glucose.



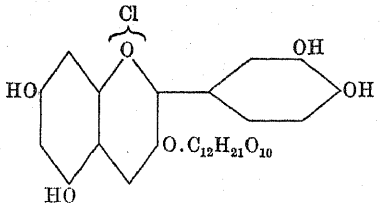
(I)



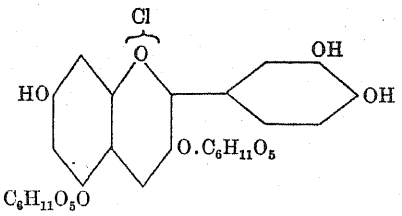
(II)

The methods we have employed throw little light on the nature of the carbohydrate groups in the anthocyanins, but in most cases we can give the position of attachment as the result of comparisons with pure natural or synthetic anthocyanins of known constitution. The simplest type of anthocyanin is represented by chrysanthemin chloride (II) which is cyanidin 3-monoglucoside.

The diglycosides can apparently be placed in one of two groups; either they are 3-biosides like mecocyanin (III) or they are 3 : 5-dimonosides like cyanin (IV). The arguments on which the constitution of cyanin are based will be fully described in papers of the series "Experiments on the Synthesis of Anthocyanins," published in the *Journal of the Chemical Society*. The evidence indicates that the 3-monosides are the basis of the anthocyanins, the other groups being subsequently attached to the molecule. Thus no 5-monoside is known. Furthermore this view affords some explanation of the changes in sugar-position type which occur, for example, in the sweet peas, the gladioli and the carnations.



(III)



(IV)

In addition, the carbohydrate residues may be acylated in the class of complex diglycosides.

Some interesting anthocyanins, not yet isolated in substance, have been encountered. The pelargonidin analogue of mecocyanin is rather widely distributed, it occurs *inter alia* in orange-red poppies, scarlet cineraria, gloxinia, tropaeolum and red-flowered anemones. Complex diglucosides of pelargonidin and delphinidin have been studied in detail by Willstätter and Mallison [1915], by Willstätter and Mieg [1915], by Karrer and Widmer [1927], and by Kondo [1931]; we have found a complex diglycoside of cyanidin in the leaves of *Coleus* and a complex diglycoside of malvidin in the flowers of *Salvia virgata nemorosa*.

Experimental.

Nature of petunidin chloride. Some comparisons of petunidin chloride with synthetic 3′-*O*-methyldelphinidin chloride were carried out by Bradley, Robinson and Schwarzenbach [1930], and no serious divergences were noted but an equimolecular mixture of delphinidin and malvidin chlorides gave almost identical results. It is remarkable that we have since found this to be true of many distribution-ratio experiments. A small specimen of finely crystallised petunidin chloride, for which we are greatly indebted to Prof. Willstätter, was available and the following experiments were carried out.

Malvidin chloride (4·002 mg.) was dissolved in 30 cc. of ethyl alcohol (1000 cc. of which contained 1 cc. concentrated hydrochloric acid) and the solution made up to 250 cc. by means of 1 % hydrochloric acid. This solution (30 cc. at 21·5°) was placed in a stoppered cylinder and shaken with air; 10 % aqueous sodium hydroxide (25 cc.) was then introduced, the liquid shaken once and immediately acidified with hydrochloric acid (10 cc.). The recovered anthocyanidin was taken up in amyl alcohol and colorimetrically estimated, using a standard prepared from malvidin chloride (0·860 mg.), ethyl alcohol (10 cc. containing hydrochloric acid as above) and amyl alcohol to 100 cc. The recovery was 78 %. Now, on performing this experiment with a solution of petunidin chloride (4·012 mg.) the recovery was quite small and indicated less than 10 % of malvidin in the sample. The recovery from both delphinidin and 3′-*O*-methyldelphinidin was less than 5 %. Using equivalent solutions of delphinidin chloride and malvidin chloride it was found that a mixture of 28 cc. of the delphinidin solution and 2 cc. of the malvidin solution gave slightly greater recovery than the natural petunidin chloride. A mixture of 7 % malvidin chloride and 93 % 3′-*O*-methyldelphinidin chloride could not be distinguished in its behaviour in the above experiment from natural petunidin. Various further tests were made with the remainder of the solutions and no divergences were noted. For example, the "93 %" and the "natural" solutions were each mixed with an equal volume of aqueous sodium acetate; the violet solutions were identical. On now shaking with 6 volumes of amyl alcohol the colour base separated as a violet film at the interface and in each case this exhibited a beautiful steel-blue reflex. In view of the facts that Willstätter and Burdick [1916] showed that petunidin yields delphinidin on demethylation and has approximately the correct methoxyl content, the only possible alternative to the methyldelphinidin hypothesis was that the substance might be an equimolecular mixture of delphinidin and malvidin. The experiments now recorded dismiss this possibility and the correctness of our conclusions was confirmed by comparisons of the extraction of equivalent solutions (50 cc.) of the "natural" petunidin chloride and of 3′-*O*-methyldelphinidin chloride (in 1 % hydrochloric acid) by successive portions (15 cc.) of the amyl ethyl ether-anisole-picric acid reagent for delphinidin mentioned below. The course of the extraction was very similar in the two cases; the pigment was gradually

removed without that sharp distinction between consecutive extracts which is characteristic of mixtures of malvidin and delphinidin. As anticipated, the first extract from the "natural" was a little more deeply coloured than that from the "synthetic"; otherwise the graded series were identical.

Tests on the original solution. The observations of the colour and of the change, if any, on heating have already been mentioned. The colour of a dilute solution of cyanin chloride is taken as standard in speaking of orange-red or blue-red.

If the colour is orange-red a small volume of the solution should be diluted with alcohol; peonidin derivatives then become much bluer and to a much greater extent than pelargonidin derivatives. In this, as in many other cases, comparison with authentic specimens is essential, and in the absence of crystallised homogeneous anthocyanins certain flower extracts will serve. For pelargonin the scarlet pelargonium; for peonin, a purified (see below) extract from the deep red peony; for cyanin, the blue cornflower; for malvin, *Epilobium* or the red-violet annual *Clarkia elegans*; for mecocyanin, any bluish-red poppy; for pelargonidin 3-diglycoside, orange-red nasturtiums; for chrysanthemin, crimson carnations; for callistephin, scarlet carnations; delphiniums and violas will give a supply of delphinidin derivatives. A portion of the original solution is shaken with amyl alcohol in order roughly to estimate the distribution number; a second extract should also be made in the case of anthocyanins of high distribution number. In the latter case, also, a test must be made for complex diglycosides. The solution is boiled in a test-tube to expel air and, whilst hot, 20 % aqueous sodium hydroxide is added drop by drop, until the colour changes through green to yellow or brownish-yellow. After actually boiling for a few seconds, concentrated hydrochloric acid is carefully added to the hot solution until the reconstituted anthocyanin indicates that the solution is acid; one more drop is then added and after 15 seconds the liquid is cooled and the distribution number again observed. In the case of monoglycosides there is no change and, although anthocyanidin is not produced under the conditions described, a second shaking is carried out for confirmation. In the case of complex diglycosides the distribution falls almost to zero.

The colour obtained on the addition of sodium acetate to the original solution is observed; this is not affected by the presence of anthoxanthins. The colours given by the pure anthocyanins are the following: callistephin, rather dull brownish violet-red; pelargonin, bright bluish-red; peonidin 3-glycosides, similar to callistephin but brighter; peonin, red-violet; cyanin, violet; mecocyanin (chrysanthemin), violet-red; malvin, bright violet; oenin, dull violet; delphinidin glycosides, blue-violet to blue. The reaction is subject to much interference, for example, from the presence of iron and/or tannin

In the case of quite clean extracts it may be desirable to test the response to the ferric reaction. Sodium carbonate is gradually added until the colour changes towards violet or blue; acid conditions are then restored by the addition of just sufficient 0·5 % hydrochloric acid. A positive reaction on the

addition of a drop of neutral ferric chloride is a deep violet coloration, replacing and more intense than the anthocyanin red. Any residual red tinge represents a negative result and the violet must be pure-toned and free from green or brown.

Examination of the anthocyanidin.

In some cases it may be necessary to purify the anthocyanin before submitting it to hydrolysis, but normally this is not the case. The solution is mixed with rather less than an equal volume of concentrated hydrochloric acid and boiled for 30 seconds (unless experiment indicates that a longer period is required). The solution is extracted with amyl alcohol and the upper layer separated and washed with water and then with 1 % hydrochloric acid. A small volume of the 1 % acid is then added in order to take up the oxonium salt after the addition of much benzene. The amount of benzene necessary to decolorise the upper layer will give some information; delphinidin requires 3–4 times the volume of the amyl alcohol; petunidin about 4–5 volumes; cyanidin, 5–6 volumes; malvidin, 5–6 volumes; peonidin, 8–9 volumes; and pelargonidin, 10–11 volumes. These figures are approximations and refer to 1 volume of the amyl alcohol solution with 3 volumes of 0·5 % hydrochloric acid. The filtered anthocyanidin solution is again extracted by amyl alcohol and the process repeated; finally, the aqueous solution of the anthocyanidin is washed three or four times with a large excess of benzene in order to remove traces of amyl alcohol.

The colour of this solution compared with that of authentic specimens gives valuable information. The following tests are carried out. A small portion is extracted with amyl alcohol and sodium acetate added; after observation a drop of ferric chloride is added and the tube gently shaken. A portion is shaken with an equal volume of a mixture of cyclohexanol (1 vol.) and toluene (5 vols.) (the cyanidin reagent); the concentration of anthocyanidin in this test should not be lower than 4 mg. in 100 cc. The upper layer is decanted into a narrow tube for observation.

A portion (concentration about 3–4 mg. in 100 cc.) is shaken with air and half its volume of 10 % sodium hydroxide added. This is immediately followed by concentrated hydrochloric acid and amyl alcohol. The recovery of anthocyanidin in this "oxidation" test is noted.

A portion (or several portions of different concentrations) is shaken with a 5 % solution of picric acid in a mixture of amyl ethyl ether (1 vol.) and anisole (4 vols.) (delphinidin reagent). (Note on recovery: amyl ethyl ether and anisole are difficult to separate by distillation, so that it is convenient to use the Abbé refractometer. The volumes % and the refractive indices are connected by a relation that is sufficiently linear for practical purposes and the following data may be of service—anisole, n, 1·5161; 4 : 1-mixture, n, 1·4902, ethyl amyl ether, n, 1·3902.)

The various anthocyanidins exhibit the following behaviour:

Pelargonidin. Amyl alcohol-sodium acetate, violet-red; no change with

ferric chloride; largely extracted by the cyanidin reagent and completely by the delphinidin reagent; not destroyed in the oxidation test. Pelargonidin is recognised by the colour of its acid solution and by the colour reactions of the anthocyanins derived from it.

Peonidin differs chiefly from pelargonidin in the colour of its acid solutions and in the colour reactions of related anthocyanins.

Cyanidin gives a reddish-violet solution when sodium acetate is added to the amyl alcohol extract over water and ferric chloride changes the violet to a bright blue colour. This is apt to be confused with malvidin containing a trace of ferric-reacting anthocyanidin. Cyanidin is fairly stable in the oxidation test; it imparts a rose-red colour to the cyanidin reagent (malvidin, a very weak mauve) and its extraction by the delphinidin reagent is not complete from dilute solutions. In order to distinguish cyanidin from impure malvidin it is best to perform the ferric reaction without sodium acetate using a carefully washed amyl alcohol solution diluted with ethyl alcohol. It is only when the anthocyanin is a 3 : 5-dimonoside that confusion with malvidin is a possibility; the reactions of cyanidin 3-glycosides are characteristic.

Malvidin gives a slightly bluer violet in the amyl alcohol-sodium acetate test than cyanidin and ferric chloride does not change it. Pure malvidin is, however, of rare occurrence. The oxidation test leaves malvidin largely unchanged and it is not extracted by the cyanidin reagent, but completely by the delphinidin reagent.

Petunidin gives a violet-blue in the amyl alcohol-sodium acetate test and pure blue after the addition of ferric chloride. It is destroyed in the oxidation test, not extracted by the cyanidin reagent and has a lower distribution than cyanidin in the delphinidin reagent. It is best recognised by successive extractions of a solution with small portions of the delphinidin reagent.

Delphinidin gives a blue solution in amyl alcohol on the addition of sodium acetate; it is destroyed in the oxidation test, not extracted by the cyanidin reagent or by the delphinidin reagent.

Hirsutidin is rare and if it occurs in any of the solutions tested it would be included under malvidin. It gives reactions similar to those of malvidin.

Colour reactions of the anthocyanins.

Pelargonin (and possibly other pelargonidin 3 : 5-dimonosides) is easily recognised. It gives a violet coloration with aqueous sodium carbonate and this becomes greenish-blue on the addition of acetone. Decisive confirmation is obtained by addition of $\frac{1}{4}$ volume of concentrated hydrochloric acid to the solution and boiling for $\frac{1}{2}$–1 minute; then on extracting with amyl alcohol a green fluorescence due to pelargonenin will be observed.

Pelargonidin 3-glycosides, for example, callistephin, give a violet-red coloration with sodium carbonate and this is rather stable towards sodium hydroxide. No other anthocyanin-type gives a similar reaction.

Peonin (3 : 5-type) gives a blue coloration with sodium carbonate. Peonidin 3-glycosides do not occur in the sequel, but the sodium carbonate reaction is a rich violet unchanged by sodium hydroxide.

Cyanin gives a rich pure blue coloration with sodium carbonate, unstable to sodium hydroxide, whereas mecocyanin (chrysanthemin) gives a blue-violet with sodium carbonate changing to pure blue with sodium hydroxide.

Malvin (3 : 5-type) gives a bright greenish-blue with sodium carbonate, whilst oenin (malvidin-3-glucoside) gives a blue-violet unchanged by sodium hydroxide.

The delphinidin glycosides have not been distinguished with the aid of their colour reactions in the absence of synthetic standards.

Very frequently the anthocyanin had to be purified before satisfactory reactions could be obtained.

Solutions of diglycosides could be exhaustively extracted with amyl alcohol and occasionally this was sufficient. Otherwise the pigment was taken up in amyl alcohol-acetophenone (2 : 1) in the presence of picric acid. This reagent was introduced by Willstätter and Schudel [1918]. The separated, filtered organic layer was agitated with 1 % hydrochloric acid and diluted with ether. Picric acid was then carefully removed from the aqueous solution by further extraction with ether. Monoglycosides were similarly purified by extraction with cyclohexanone and picric acid or with ethyl acetate and picric acid; the extracts were precipitated with light petroleum and the anthocyanins taken up in 1 % hydrochloric acid. The solution was usually extracted with benzene, then with cyclohexanone and again with benzene. Occasionally the process was repeated.

Summary of results.

Achillea Kelwayi. Complex diglycoside.

Aconitum napellus. Delphinidin diglycoside; contains much anthoxanthin.

Aesculus hippocastanum. The pink flowers contained a cyanidin 3-glycoside which required exhaustive purification. The distribution was intermediate suggesting a rhamnoglucoside or the like.

Althaea rosea. Various hollyhocks were examined and the results suggested mixtures of malvidin, petunidin and delphinidin mono- and di-glycosides. Delphinidin and malvidin were present beyond doubt and the occurrence of petunidin was inferred from the behaviour of the anthocyanidin on successive extraction with the delphinidin reagent.

Anchusa italica (Dropmore var.). The mauve buds and blue flowers gave the same results. The anthocyanin is essentially a delphinidin diglycoside, but the behaviour of the anthocyanidin with the delphinidin reagent indicated some methylation of the pyrogallol nucleus.

Anemone coronaria (St Brigid var.). Red flowers contained a pelargonidin 3-bioside and violet flowers contained cyanidin 3 : 5-dimonoside, possibly mixed with a malvidin derivative. The anthers of both varieties contain what appears to be the same anthocyanin characterised by a high distribution number. Further examination of the anemone is projected; this flower was studied before our system had been adequately developed.

Armeria maritima. Malvidin 3 : 5-dimonoside.

Arum maculatum. Anthocyanin absent.

Astilbe gloria purpurea. The reactions were much obscured by other substances but the anthocyanin proved to be a cyanidin 3-glycoside. The distribution was intermediate between that of mecocyanin and that of chrysanthemin.

Aubrietia deltoides. The purple flowers of the variety "Dr Mules" were found to contain a delphinidin diglycoside, whilst the rose-red Leichtlini was found to contain a cyanidin 3:5-dimonoside.

Begonia semperflorens. A fibrous-rooted bedder with crimson flowers gave a solution containing a cyanidin 3-bioside; the leaves of another variety with small pink flowers contained cyanidin 3-bioside.

Tuberous-rooted begonias have also been examined; the scarlet-flowered varieties contain pelargonidin 3-bioside and the crimson kinds, cyanidin 3-bioside.

Bellis (Rob Roy). A co-pigment makes the solution much too blue-red, but the anthocyanin is a cyanidin 3-bioside.

Berberis aquifolium. The deep purple fruits contain 3-monoglycosides of malvidin, delphinidin and probably also, petunidin. The anthocyanin gives a violet coloration in aqueous sodium carbonate and this becomes green on the addition of sodium hydroxide. Cyanidin is absent, but the reactions cited are those of cyanidin 3-glycosides, and it is evident that these changes may be observed in the delphinidin series also.

Buddleia amplissima. Delphinidin diglycoside.

Calceolaria. A magenta garden variety contained cyanidin 3-bioside and this was the main anthocyanin of a brown-red kind; in this case the cyanidin was partly replaced by pelargonidin.

Callistephus hortensis. A blue-violet Aster of the "Victoria" class was found to contain a delphinidin diglycoside. The characteristic pigments of other varieties are monoglycosidic. According to Fleming [1929] no true blue aster exists.

Campanula glomerata. Purplish-blue flowers contained a delphinidin diglycoside mixed with a small proportion of a reddening pigment. The variety "Telham Beauty" has blue-violet flowers and the anthocyanin was a delphinidin diglycoside.

Pink flowers of *Campanula Medium* were unexpectedly found to contain a pelargonidin 3-bioside; this anthocyanin is usually associated with a scarlet-coloured flower, the rose-pinks based on pelargonidin owing their colour to pelargonin or similar anthocyanin.

Carduus lanceolata. Cyanidin 3:5-dimonoside.

Centaurea nigra. The distribution number was zero, otherwise all tests showed that the anthocyanin was a cyanidin 3:5-dimonoside.

Chaerophyllum anthriscus. The purple leaves gave an extract containing a cyanidin 3:5-dimonoside.

Charies heterophylla. Delphinidin diglycoside.

Chrysanthemum coronarium. Pyrethrums such as "Countess Poulett," "Sutton's Blue Red," and "Mrs James Leake" were all found to contain a cyanidin 3-glycoside. There was a powerful co-pigment effect.

Chrysanthemum tricolor gave solutions having exactly the same properties.

Cichorium entybus. Delphinidin diglycoside.

Clarkia elegans. A coral pink variety yielded pelargonidin 3:5-dimonoside and a violet-flowered variety gave a very clean solution of pure malvidin 3:5-dimonoside. In this case the malvidin was free from any trace of a substance giving a positive ferric reaction.

Clematis Jackmani. The anthocyanin is a delphinidin diglycoside mixed with a relatively small proportion of methylated delphinidin derivatives.

Coleus Blumei. The leaves of several varieties were examined and all found to contain a complex diglycoside of cyanidin. The acid solution was very blue-red and developed a fine blue colour on the addition of sodium acetate.

The distribution was monoglucosidic, or even higher, and after purification the anthocyanin gave a reddish-violet with sodium acetate. The diglycoside resulting from the hydrolysis of the anthocyanin (cold sodium hydroxide in a hydrogen atmosphere) was purified and its distribution between butyl alcohol and 1 % hydrochloric acid was identical with that of cyanin. The whole of the reactions of the diglycoside supported the view that it is actually cyanin. So that coleusin is an acyl derivative of cyanin and the nature of the acid is being investigated.

Convolvulus sepium. Rose-heliotrope flowers contain a cyanidin 3-monoglycoside.

Crataegus oxyacantha fl-pleno rosea. The flowers are coloured by a cyanidin 3-bioside and the skins of the red berries contain a cyanidin 3-glycoside.

Dactylis glomerata L. The bluish-mauve flowers were separated and a cyanidin 3-bioside was diagnosed. The distribution is, however, intermediate between monoglucosidic and diglucosidic.

Delphinium. A number of the garden perennial varieties have been examined, for example, "Prince of Wales," "True Blue," "W. T. Ware," "Corry Ware," "Mrs Townley Parker" and *D. formosum*; also annuals derived from *D. consolida.* All gave evidence that delphinidin is the main anthocyanidin, but it is mixed in most cases with a small proportion of a methylated derivative.

The anthocyanins all exhibited the stability in aqueous sodium carbonate solution, which, Willstätter and Mieg [1915] showed to be characteristic of delphinin.

Dianthus barbatus. The bluish-red flowers of the type gave a blue-red acid extract and after exhaustive purification of the anthocyanin it was characterised as cyanidin 3-monoglycoside. The garden varieties (Sweet William) also furnished 3-monoglycosides; a crimson and also a black-crimson (*nigricans*) variety furnished a solution which could not be distinguished in distribution number tests from one of chrysanthemin chloride. A scarlet variety, however, was found to owe its colour to a pelargonidin 3-monoglycoside, probably callistephin and following that anthocyanin exactly in distribution number tests. These apparent monoglycosides were found to be such in fact, and do not owe their high distribution number to complex diglucosidic constitution.

Dianthus caryophyllus. We are greatly indebted to Mr E. M. Morgans for supplies of a number of varieties of carnations.

"Nigger" and "Topsy" (deep brown-red) owe their colour to a cyanidin 3-monoglycoside, the distribution number of which, after purification, was identical with that of chrysanthemin under the same conditions. "Brilliant," "Aviator" and "Spectrum" (scarlet) are similarly coloured by a pelargonidin 3-monoglycoside tallying with callistephin. "Mary Allwood" (salmon-red) also contains the pelargonidin 3-glycoside. But, surprisingly, "Improved Ward" (bluish-pink) contains a pelargonidin 3 : 5-dimonoside agreeing in distribution number (*n*-butyl alcohol) with pelargonin.

Dierama pulcherrima. Mauve-rose flowers contained a malvidin 3 : 5-dimonoside.

Digitalis purpurea. The anthocyanin is a cyanidin 3 : 5-dimonoside.

Dodecatheon integrifolia. Malvidin 3 : 5-dimonoside. The solutions contained much anthoxanthin.

Epilobium angustifolium and *E. hirsutum.* The willow herbs contain a malvidin 3 : 5-dimonoside and apart from the presence of a co-pigment these available flowers provide good standards for malvin-type reactions.

Erigeron speciosus. A very strong co-pigment effect was noted; the anthocyanin is a delphinidin diglycoside.

Fagus sylvatica. Leaves of the copper beech give blue-red extracts and were found to contain a complex cyanidin 3-bioside. The distribution number of this anthocyanin closely approaches that of chrysanthemin for amyl alcohol, ethyl acetate in presence of picric acid and butyl alcohol, all with 1 % hydrochloric acid. A considerable divergence was, however, noted when the organic solvent was *n*-butyl alcohol mixed with $\frac{1}{3}$ vol. of benzene. The beech anthocyanin had the higher distribution number. The complex nature of the pigment was proved in the usual manner and an attempt to identify the acid concerned will be made in due course. This is the first example of a complex diglycoside derived from a 3-bioside anthocyanin.

Fragaria vesca. The colour of strawberries is due to a pelargonidin 3-monoside.

Fuchsia. The purple petals of the inner corollae of several varieties (for example, "Rose of Castile") gave identical results which have already been described. The anthocyanin is mainly a malvidin 3 : 5-dimonoside but the alkali-colour reactions were not quite standard, being too red even after purification of the pigment through the picrate. The anthocyanidins concerned could be pelargonidin and peonidin only, since the ferric reaction was quite negative.

A clue was obtained by studying the bluish-red outer petals which were found to contain a peonidin 3 : 5-dimonoside mainly, mixed with a blueing component. We then found that the reactions could be perfectly matched by adding a little peonin to malvin for the inner petals or a little malvin to peonin for the outer petals. The use of pelargonidin 3-bioside from *Papaver orientale* gave a very poor match and pelargonidin 3 : 5-dimonoside cannot be present, since no pelargonenin-type fluorescence could be observed. The absence of pelargonidin from the fuchsia

anthocyanidins was confirmed by adding saturated aqueous picric acid (1 vol.) to a solution in 0·5 % hydrochloric acid (1 vol.) and shaking with a mixture (1 vol.) of *iso*propyl ether with 3 vols. of *iso*amyl ether. The upper layer was not coloured by the anthocyanidin, but after adding a few drops of a dilute solution of pelargonidin chloride and again shaking, the organic layer was coloured by anthocyanidin.

Carrying out the same experiment but using a mixture of equal volumes of *iso*propyl ether and *iso*amyl ether, the presence of peonidin was indicated in the anthocyanidins from both types of petals. This mixture distinguishes peonidin from malvidin in dilute solutions; in the former case the upper layer assumes a bluish salmon-red colour but malvidin is not extracted, although the picrate may be precipitated. The variety "Corallie" with long, tubular, salmon-red flowers was found to contain a pelargonidin 3 : 5-dimonoside.

Galega officinalis. The violet flowers contain a delphinidin diglycoside.

Geranium pratensi. Malvidin 3 : 5-dimonoside.

Geranium Robertianus. The leaves and stem furnished an extract of a cyanidin 3-bioside, whilst the bluish-pink flowers contained a malvidin 3 : 5-dimonoside.

Geum chiloënse. The varieties "Mrs Bradshaw" and "Orange Queen" gave the same results. The anthocyanin is a cyanidin 3-monoside but there are indications of the presence of a small proportion of a pelargonidin derivative.

Gladiolus gandavensis. This group exemplifies well our thesis that colour variations are usually due to the appearance of different anthocyanins. The salmon-pink varieties all contain a pelargonidin 3 : 5-dimonoside. In some cases, for example, "Flaming Sword," the alkali-colour reactions suggest admixture with a small proportion of a peonidin or cyanidin derivative. In the scarlet sorts, for example, "Scarletta," we find a pelargonidin 3-bioside. The distribution number is lower than that of callistephin but is higher than that of the pelargonidin bioside in the gloxinia and scarlet cineraria.

A crimson-flowered variety contained an anthocyanin, the distribution number of which was zero; it gave all the reactions of a cyanidin 3 : 5-dimonoside. The occurrence of a malvidin 3 : 5-dimonoside was noted in a purple-red variety (Jacob von Bengeren); this contained a trace of an anthocyanin giving a positive ferric reaction.

G. primulinus, a red-violet variety, was also found to contain a malvidin 3 : 5-dimonoside.

Gloriosa vivescens Rothschildiana. Specimens kindly supplied by Lord Rothschild were found to contain cyanin, which in this case was isolated and identified. There is also a bright yellow anthoxanthin.

Gloxinia. "Reading Scarlet" and "Beacon" contain a pelargonidin 3-bioside, whilst "King George" is coloured by the same pigment together with a small proportion of an anthocyanin giving more blue-red acid solutions. "Purple Sultan" and "Violet" yield diglycosidic anthocyanins based on malvidin but containing some cyanidin, petunidin or delphinidin derivative. The alkali-colour reaction was not precisely of malvin-type and it may be that this is due to the rare malvidin 3-bioside constitution or more probably to lack of homogeneity.

Hyacinthus orientalis. "King of the Blues" contains a delphinidin diglycoside and "Queen of the Pinks" contains a pelargonidin 3 : 5-dimonoside. Neither anthocyanin is homogeneous, the evidence being in favour of the presence of a cyanidin derivative in both violet-blue and pink flowers.

Hydrangea hortensis. Considerable interest attaches to a study of the pigments of these flowers on account of the popular idea that iron in the soil helps in the development of the blue colour. Unfortunately the blue kinds do not produce their pigment lavishly, but we have been able to form an opinion respecting the relation between the respective colouring matters.

A red-flowered variety, "Parzival," gave a bluish-red solution containing a co-pigment and the anthocyanin was a delphinidin derivative. As the distribution number seemed rather high for a delphinidin diglycoside, tests for complex diglycosidic character were made and these gave a positive result, the recovered diglycoside having a distribution number approximating to zero. The anthocyanin from a blue-flowered variety, "Maréchal Foch," had a much lower distribution number than that from the red flowers. It proved to be a delphinidin diglycoside. It is noteworthy that the colour from the red flowers is sensitive to co-pigments, whereas that from the blue flowers is much less so.

Iberis umbellata. The purple flowers contain a cyanidin 3 : 5-dimonoside mixed with a relatively small proportion of a malvidin derivative.

Ilex aquifolium. The skins of holly berries contain a pigment which tallies best with a pelargonidin 3-bioside; a further examination is necessary.

Iris Kaempferii. A bluish-purple variety of the Japanese Iris contained a malvidin 3 : 5-dimonoside mixed, probably, with a petunidin derivative. Several unnamed blue and violet varieties of the German Iris furnished solutions of delphinidin diglycoside much blued by co-pigments and the variety "Imperator" gave identical results.

Ixia crateroides. The cerise-coloured flowers contain a cyanidin 3 : 5-dimonoside and this is probably cyanin, since a comparison of the purified pigment with cyanin in respect of its distribution number (*n*-butyl alcohol—1 % hydrochloric acid) showed that these were identical.

Lathyrus. L. grandiflorus, crimson flowers, develops a mixture of diglycosidic anthocyanins; the anthocyanidins consist of malvidin (less than 40 %) and delphinidin, whilst the presence of petunidin may be inferred from the behaviour of the anthocyanidin solution on extraction with successive small volumes of the delphinidin reagent. *L. latifolius* is coloured by nearly homogeneous malvidin 3 : 5-dimonoside. *L. odoratus* provides one of the most interesting groups and we propose to study it in some detail; the solutions are clean and the petals are easily extracted.

For named varieties of Sweet Peas as also for Gloxinias, Cinerarias, Salpiglossis, *etc.*, we are very greatly indebted to Messrs Sutton and Sons of Reading. "2 LO," scarlet, and "Glorious," pink, gave solutions of a pelargonidin 3-bioside. "Crusader," bluish-pink, contains, however, pelargonidin 3 : 5-dimonoside. "Captain Blood," blood-red, gives solutions containing more anthoxanthin than usual in the group. Its chief anthocyanin is cyanidin 3-bioside, but this is mixed with a small proportion of pelargonidin 3-bioside. Cyanin and pelargonin types of anthocyanins were not present. "Damask Rose," bluish-red, contains a mixture of pelargonidin and cyanidin 3 : 5-dimonosides. The same is true of "Sybil Henshaw," bluish-red, but in this case the proportion of cyanidin derivative is higher than in "Damask Rose." A match for all the reactions was obtained using 70 % of cyanin and 30 % of pelargonin. No match could be made using mecocyanin. Doubtless a variety yielding good cyanin-type reactions free from pelargonin exists but, even although we have not yet encountered it, we consider the proof of the occurrence of cyanidin 3 : 5-dimonoside is satisfactory.

It thus appears that the Sweet Peas contain both types of pelargonidin and cyanidin diglycosides and these are by no means all. "Bacchus," wine-red, possesses colouring matters based on methylated delphinidins, delphinidin itself appears to be absent. Cyanidin is also absent and we consider that the chief anthocyanins are malvidin and petunidin 3 : 5-dimonosides. The evidence for malvidin is the result of the oxidation test and the behaviour towards the delphinidin reagent; petunidin is indicated by the latter and by the positive ferric reactions, cyanidin and delphinidin being ruled out.

"Chieftain," light red-purple, contains chiefly malvidin 3 : 5-dimonoside and the same is true of "Royal Purple," red-purple; these two give weak ferric reactions as purified anthocyanin or as anthocyanidin. "The Flag Lieutenant," purple, and "Black Diamond," deep red-purple, furnish anthocyanins giving a strong ferric reaction; malvidin and delphinidin were proved to be present and cyanidin was absent. The existence of petunidin in the mixture is strongly suspected but we cannot put this forward with confidence. The anthocyanins are all diglycosidic and the alkali-colour reactions are of the malvin type.

"Blue Bird," violet-blue, contains a delphinidin diglycoside as the major anthocyanin.

Lavandula vera and *L. nana* both contain a delphinidin diglycoside.

Lavatera trimestris rosea splendens. This anthocyanin will be further examined; it appears to be based on petunidin. The oxidation test on the anthocyanidin shows that cyanidin and malvidin are absent, yet the ferric reaction is positive and the distribution with the delphinidin reagent is approximately correct for petunidin. Peonidin and pelargonidin are absent. Moreover, the reactions of the anthocyanin, which has a high distribution number, agree with the suggestion that it is petunidin 3-monoside. We have not excluded the complex diglycoside possibility in this case.

Lilium Martagon. The reddish-purple flowers and also the stems yielded a blue-red solution containing a co-pigment. The anthocyanin is a cyanidin 3 : 5-dimonoside.

Lilium umbellatum (Dahurian Lily) has orange flowers but the anthocyanin is a cyanidin

3-bioside. It required exhaustive purification on account of the presence of much anthoxanthin and other substances.

Linum. A crimson-scarlet-flowered annual variety (*grandiflorum* var. *rubrum*) was found to contain a delphinidin diglycoside. In this case we may be justified in stating that the anthocyanin is a 3-bioside, since the coloration in sodium carbonate is royal blue and not greenish-blue as usual. There seems to be little co-pigment in this case and for these reasons we propose to attempt the isolation of this anthocyanin. It is remarkable that a flower so far removed from blue should contain an anthocyanin based on unmethylated delphinidin. Nevertheless, a little cyanidin appears to be present.

Linum narbonense, blue, contains a delphinidin diglycoside and, by contrast with the preceding, this is a 3 : 5-dimonoside and gives a greenish-blue coloration in sodium carbonate; also the colour in sodium acetate is violet-blue, whereas in the case of the annual scarlet flax it was violet.

Lupinus polyphyllus. The varieties "Pink Perfection," "H. Marshall," reddish-violet, and "Downers Delight," dull brownish salmon-red, contained a cyanidin 3-bioside. "King of the Blues," reddish-blue, contains a delphinidin complex diglycoside; "Sutton's Saxe Blue," "Sutton's Ruby" and "Amethyst" from Brookside Nurseries, Oxford, give quite similar results. "Victoria," bluish-dark purple, is also a complex diglycoside and the results suggest that it is a petunidin derivative. This conclusion is provisional.

Lychnis chalcedonica. The anthocyanin is a pelargonidin 3-glycoside with a distribution number intermediate between callistephin and pelargonidin 3-bioside.

Lythrum salicaria. "Brightness" contains a malvidin 3 : 5-dimonoside and a trace of an anthocyanin having a positive ferric reaction.

Matthiola incana. Some single and double ten-week stocks have been examined and all the anthocyanins therein have high distribution numbers and were found to be complex diglycosides. A magenta single-flowered variety furnished an acyl derivative of a pelargonidin 3 : 5-dimonoside; this had a distribution number > 50. A violet-coloured variety contained an acylated cyanidin 3 : 5-dimonoside. A deep purple double-flowered kind furnished an anthocyanin with a distribution number nearer to that characteristic of a diglycoside but still too high. On hydrolysis this yielded an anthocyanin not to be distinguished in distribution and other tests from cyanin.

It is noteworthy that the solutions of all these complex diglycosides of *Matthiola* are much too blue-red and that heating the solutions has a relatively small effect. We consider that in these cases the attached phenolic acid residue exerts an intramolecular effect similar to the intermolecular effect of the co-pigments. Coleusin is under all circumstances much bluer red in solution than cyanin.

Myosotis dissitiflora. "Blue King" contains a delphinidin diglycoside.

Nepeta mussini contains a complex diglycoside of delphinidin; the only difficulty was that the sodium acetate-amyl alcohol test on the anthocyanidin gave rather too red a shade. Other tests, however, confirmed the diagnosis of delphinidin.

Nepeta ókrani also contains a complex diglycoside of delphinidin, but in this case the evidence of contamination is still more definite; also the distribution number of the original anthocyanin is higher than in the case of that from *N. mussini*.

Nemophila insignis. Delphinidin diglycoside.

Nigella damascena. Malvidin 3 : 5-dimonoside.

Oenothera amoena (syn. *Godetia rubicunda*). A bluish-red variety gave diglycosidic anthocyanins, certainly 3 : 5-dimonosides, but apparently not homogeneous and derived from malvidin and cyanidin (or petunidin).

Origanum vulgare. The anthocyanidin is very definitely malvidin and it is found unmixed with other anthocyanidins; further the anthocyanin is diglycosidic. The characteristic malvin-type alkali-colour reaction could not be obtained and the colour with sodium carbonate was violet, but this is possibly due to impurities not removed in the cyclohexanone-picric acid process.

Papaver orientale bracteatum. The orange-scarlet flowers, after removing all the dark central parts, gave a solution of a pure pelargonidin 3-glycoside. The colour reactions matched those of callistephin. The distribution number, however, was zero and the distribution number with solvents other than amyl alcohol was also abnormally low. Perhaps this anthocyanin is a trioside. The epidermis of the dark portions gave a solution of cyanidin 3-bioside, doubtless mecocyanin

and the anthers also contained the cyanidin derivative. A pink variety, "Queen Alexandra," also contained a pelargonidin 3-bioside and the explanation of the colour change is probably to be found in the presence of a co-pigment.

Many other varieties of poppies have been examined and in general the pink and scarlet kinds (also the orange, *Papaver nudicaule*) contain pelargonidin derivatives, always 3-glycosides, whilst the deep red and blue-red varieties contain a cyanidin 3-bioside (mecocyanin). The darker portions are never free from mecocyanin and this may, or may not extend to other parts of the petals. A common form of the field poppy (*P. rhoeas*) has scarlet flowers, the petals of which (excluding the splashes) yield solutions containing both pelargonidin and cyanidin derivatives. Pink Shirley poppies give blue-red acid solutions, but these are co-pigmented and purification of the anthocyanin through the picrate yields normal pelargonin-like solutions.

Mecocyanin [Willstätter and Weil, 1916] may, as usually prepared, be accompanied by an impurity; its alkali-colour reaction can be matched by a solution made by adding about 12 % of cyanin to chrysanthemin. On the other hand, this tendency to give slightly bluer reactions than chrysanthemin may be an inherent property of the substance.

Pentstemon. "Southgate Gem" and *isophyllis* gave scarlet-red solutions having similar reactions; a strong co-pigment was present. The anthocyanin is a pelargonidin 3 : 5-dimonoside, the distribution number of which is higher than that of pelargonin and lower than the complex diglycosides of this group.

Petunia. In view of our section on the existence of petunidin, it is interesting to note that permanganate-coloured petunias furnished an anthocyanin of the methylated delphinidin type, but this gave, on hydrolysis, an anthocyanidin consisting of malvidin to the extent of at least 57 %. The remainder was probably petunidin, but this could not be proved in the presence of so much malvidin. Willstätter and Burdick [1916] used a special blue petunia and their process of isolation may have had much to do with the successful removal of anthocyanins based on malvidin and delphinidin.

Phlox. A few varieties of herbaceous Phlox have been examined; they are remarkable for the efficiency of their co-pigments.

"Jules Sandean," pink, contains a pelargonidin 3 : 5-dimonoside and a trace of a cyanidin derivative; "Leo Schlageter," crimson-scarlet, contains the same anthocyanin with a little more cyanidin derivative. On heating, the solutions in 1 % hydrochloric acid became much more orange-red and the blue-red colour returned on cooling. This property was not destroyed by heating with sodium hydroxide until bright yellow, boiling the solution for a few seconds, and then reproducing the anthocyanin. The solution was still blue-red and the alternation of colour on heating and cooling could still be brought about. The co-pigment is therefore not an ester. A blue-violet "Iris Phlox" was found to owe its colour to a malvidin 3 : 5-dimonoside.

Potentilla Willmottiae. Cyanidin 3-bioside; the distribution number suggests the possibility of rhamnoglucoside. Other potentillas having a like colour or deep brown-red colour, gave similar results.

Poterium obtusum. The blue-red flowers contain a cyanidin 3 : 5-dimonoside.

Primula Juliana contains a malvidin 3 : 5-dimonoside and *P. sinensis*, "Reading Ruby," also contains this type of anthocyanin.

Prunus Avium. The skins of small black cherries contain cyanidin 3-monoside; the distribution number is higher than that of keracyanin [Willstätter and Zollinger, 1916].

Prunus communis. The skins of a deep purple plum were found to contain a cyanidin 3-monoside. The variety was "Rivers Early."

Prunus Pissardii. The leaves contain a cyanidin 3-monoside; there was no complex diglycoside.

Pyrus malus. The skins of the apples "Wine Sap" and "Jonathan" were found to contain a cyanidin 3-monoside. The flowers of "Niedzwetzkyana" also yield a cyanidin 3-monoside.

Rheum rhaponticum (Sutton's rhubarb). The colouring matter in the skin of the stem is a cyanidin 3-bioside.

Rhododendron indicum. A salmon-red azalea gave an extract singularly free from anthoxanthins and the colouring matter was a cyanidin 3-monoside. It was almost certainly chrysanthemin, since its distribution numbers under four sets of conditions were identical with those of chrysanthemin. The solvents employed were amyl alcohol, butyl alcohol with 20 vols. % of benzene, ethyl

acetate in the presence of picric acid in small and also in large concentration; all were put in competition with an equal volume of 1 % hydrochloric acid.

Ribes. Willstätter and Bolton [1916] found that the berries of *R. rubrum* L. contained a cyanidin diglucoside. This is a cyanidin 3-bioside and it is accompanied by a pelargonidin or peonidin derivative. A curious feature was the difficulty experienced in getting a positive ferric reaction with the original solution.

The skins of large red gooseberries (*Ribes grossularia*) contain a cyanidin 3-monoside and the black currant (*Ribes nigrum*) contains a cyanidin 3-bioside. In the latter case there is much co-pigment in the original solution and normally coloured solutions result from purification of the anthocyanin through the picrate. Cyanidin was the only anthocyanidin found, but it should be noted that a small percentage of delphinidin could not in any case be detected and the related anthocyanin would probably be eliminated in the purification process.

Rosa. Young red leaves of the rose contain a cyanidin 3 : 5-dimonoside, doubtless cyanin.

Rubus idaeus. Willstätter and Bolton [1916] diagnosed a cyanidin glucoside in the raspberry; it is a cyanidin 3-bioside. Loganberries also contain a cyanidin 3-bioside and in both cases the reactions represent a close approach to those of pure mecocyanin.

Rubus odoratus. An interesting anthocyanin occurs in the flowers. It has a high distribution number and on hydrolysis yields a cyanidin 3 : 5-dimonoside indistinguishable in its reactions and distribution between *n*-butyl alcohol and 1 % hydrochloric acid from cyanin. This complex cyanidin diglycoside is probably not identical with that found in *Coleus Blumei*; its solutions though blue-red are not markedly so as in the latter case, and the reactions with sodium acetate are strikingly dissimilar. The anthocyanin under consideration gives a violet-red and that from *Coleus* leaves almost a pure blue coloration. The difference must reside in the nature or mode of attachment of the associated acid and further investigation is projected.

Rumex acetosa L. Cyanidin 3-bioside.

Salpiglossis sinuata. Sutton's "Large Blue-flowered" and "Light Blue and Gold" contain a delphinidin diglycoside. The reactions of the solutions from "Velvet Red" and "Black Knight" agree best with the view that they contain mainly petunidin diglycosides.

Salvia splendens. The variety "Grahamei" has crimson flowers and as the more orange-scarlet types were employed by Willstätter and Bolton [1916] it is of interest to note that this variety, also, contains a complex pelargonidin 3 : 5-dimonoside.

Salvia patens. Delphinidin diglycoside, possibly complex.

Salvia pratensi. The anthocyanin appears to be not homogeneous; it is mainly a complex malvidin 3 : 5-dimonoside, but there is also a substance present which gives a ferric reaction.

Salvia virgata nemorosa. The interesting anthocyanin is a complex malvidin 3 : 5-dimonoside; it is very blue-red in acid solution and is relatively difficult to hydrolyse by means of acids, in which respect it resembles the anthocyanin of *Coleus*. The diglycoside obtained after alkaline hydrolysis and recovery by means of hydrochloric acid is probably malvin. The acid of the complex has been isolated and is being examined.

Saponaria. A scarlet-crimson annual variety gave a solution which contained a cyanidin 3 : 5-dimonoside and very satisfactory reactions were obtained with the original unpurified material.

Saxifraga sanguinea superba. The solution was blue-red and the anthocyanin had a high distribution number. It yielded cyanidin on hydrolysis but did not tally with any of the known types.

The reaction with sodium acetate is blue-violet even after purification through the picrate, and the violet colour with sodium carbonate becomes blue on the addition of sodium hydroxide, but not so readily as in the usual chrysanthemin-type cases. There is some novel feature in connection with this anthocyanin and next season a closer investigation is proposed.

Scabiosa arvensis. Delphinidin diglycoside.

Scrophularia nodosa. This figwort contains a cyanidin 3-bioside and much anthoxanthin.

Solanum dulcamara. The purple flowers contain a delphinidin diglycoside which gives a deep blue-violet with sodium acetate and so is probably a 3 : 5-dimonoside.

Senecio (*Cineraria stellata*). Deep blue hybrids contain a delphinidin diglycoside (distribution number zero), and the aqueous acid extracts become pure blue on the addition of sodium acetate. Similar results were obtained with named varieties, for example, "Forget-me-not Blue," "Royal

Blue," "Blue Gem," "Blue with White Zone" and "Duchess Star." On the other hand, the rich purple flowers give an extract that develops a corresponding blue-violet on the addition of sodium acetate. The distribution number is still zero and the anthocyanidin is delphinidin. Possibly the change is in the attachment of carbohydrate residues. Cineraria "The King" and "Brilliant Ruby" contain the same anthocyanin which has a low distribution number (but not zero), and appears to be a malvidin derivative mixed with a substance giving a positive ferric reaction. The reactions do not, however, tally with those of known types and this, together with the blue cineraria, will be further studied. Cineraria "Pink Pearl" and the large scarlet-flowered variety contain a pelargonidin 3-bioside unmixed with other anthocyanins.

Silene angelica. The flowers of this catchfly contain a malvidin 3 : 5-dimonoside mixed with a trace of a cyanidin derivative, and the stems contain a cyanidin 3-bioside with a trace of a malvidin derivative. Attention is directed to the fact that this distribution of a malvin-type and meco-cyanin-type of anthocyanin has also been observed in the *Geranium Robertianus* (Herb Robert).

Sistus purpurea and *Sistus* "Sunset" appear to contain a cyanidin 3-bioside, but the change of colour from violet to blue on adding sodium hydroxide to a solution containing sodium carbonate was obscured by impurities not removed by the ordinary processes.

Stachys sylvatica. Cyanidin 3-monoside.

Statice sinuata. The violet-blue flowers yielded nothing to 1 % hydrochloric acid and had to be ground with the solvent in a mortar. (Note: this is not usually a good practice unless the solutions are centrifuged, when it is time-saving.) The very blue-red extract was found to contain a delphinidin diglycoside unmixed with other anthocyanins.

Tricuspidaria dependens. The reactions of the anthocyanin from this crinodendron were much obscured but the anthocyanidin is cyanidin, the distribution number was very low and after exhaustive purification the cyanidin 3-bioside reactions were observed, although not in the usual unambiguous manner.

Trifolium. "Sutton's Early Red Clover" gives an extract containing a cyanidin 3-bioside.

Tritoma uvaria. The flowers do not contain anthocyanin.

Tropaeolum majus L. Willstätter and Bolton [1916] stated that the flowers contain a cyanidin derivative; this is true of the deep brown-red variety and the anthocyanin is a cyanidin 3-bioside. The common orange-scarlet variety contains a pelargonidin 3-bioside and the anthocyanins are thus like those of the poppy.

Tulipa Gesneriana L. This is a very complex group characterised by mixtures of anthocyanins. Willstätter and Bolton [1916] found that the anthocyanin of a dark red garden tulip was cyanin, occurring both in the petals and the anthers. We are quite prepared to accept this finding (especially in view of our experiences with the sweet peas and gladiolus), but have not ourselves encountered a similar variety. We are much indebted to Sir Daniel Hall for supplies of varieties of tulips and the present is an interim report; the examination of the tulips is much more difficult than that of most other groups.

The varieties "Orange Perfection," "D," "City of Haarlem" and "Inglescombe Scarlet" constitute a group in which the main anthocyanin is pelargonidin 3-bioside, but it is mixed with some cyanidin 3-bioside in all cases. A variety "Electra" is typical of a middle group in which the main anthocyanin is a cyanidin 3-bioside and the subsidiary pigment, present in substantial relative amount, is pelargonidin 3-bioside. Other members of this group are probably "Dorothy Ann," "Couleur Cardinal," "Proserpine," "Cecilia," "28" and "Mary Swan." "Venus," "Rhoda Backhaus" and "Eos" also contain cyanidin and pelargonidin 3-biosides, but in these cases we did not gain an idea as to which predominated. At the other end of the scale "William Copland" was found to contain a delphinidin diglycoside and similar varieties are "Daphne," "Lucy Lund," "Widor" and "916." The greater number of varieties are intermediate between the cyanidin 3-bioside group and the delphinidin diglycoside group. They are free from pelargonidin derivatives and comprise "Giant," "Isobel," "Velvet King," "Orange Girl," "Julietta," "Prancing Nigger," "47," "29," "Cecil Dolling," "Lavinia," "Philip de Commines," "Grey Dawn," "Perdita," "Zulu," "La Noire," "Sultan." All are diglycosidic and none is homogeneous. The anthocyanidins derived from "La Noire" and "Sultan" have been separated in some quantity and the result of their examination is to suggest that they consist of petunidin and delphinidin with a trace of malvidin, but work on this topic is still in progress. A summary of the results is that in the

tulips we find a group in which pelargonidin and cyanidin occur as 3-biosides and then apparently with a rather sharp separation a second group containing delphinidin derivatives.

The varieties "Grey Dawn" and "Perdita" were examined in "breeder" and in "broken" condition; in both cases the extracts from the former were bluer but no great differences could be detected in the anthocyanins.

Urtica dioica. The flowers contain a cyanidin 3 : 5-dimonoside.

Verbena, Crimson hybrid. A cyanidin 5-dimonoside was diagnosed, but this case presented some curious features in that hydrolysis was difficult and the solutions were much too blue-red.

Vicia cracca. Delphinidin diglycoside.

Viola gracilis and other violas contained a delphinidin diglycoside, doubtless violanin in many cases. The causes of colour variation in this family, apart from the presence of carotenoids and anthoxanthins, appear to be methylation of the delphinidin nucleus and the presence or absence of co-pigments. Thus "Pickerings Blue" gives a very blue-red acid extract and the redder-violet *Viola cornuta* yields a redder acid solution. But on heating the two solutions are identical in tone and the colour difference returns on cooling. The co-pigment present in "Pickering's Blue" can be removed and introduced into the solution from *Viola cornuta;* the situation is then reversed. Evidently the difference in colour in this case is purely a question of the co-pigment.

In other cases of very red-violet violas it was shown qualitatively by the micro-Zeisel method that the anthocyanidin is partly methylated. Cyanidin derivatives were not encountered in the violas.

Weigela (*Diervilla hybrida,* Eva Rathké). The crimson flowers contain a cyanidin 3-monoside.

Zapania nodiflora. Delphinidin diglycoside.

Some observations on co-pigments.

The following substances have little or no effect in low concentrations on the colour of dilute acid solutions of oenin chloride: alanine, asparagine, nicotinic acid, quinolinic acid, anthranilic acid, *m*-aminobenzoic acid, *p*-aminobenzoic acid, phthalic acid, benzilic acid, glucose, raffinose, maltose, starch, inulin, methylglycoside, rutin (possibly due to sparing solubility), euxanthic acid (same comment), helicin, pyridine, 2 : 4-dihydroxyquinoline, catechol, hesperidin.

The following substances have a slight blueing effect on oenin solutions. Tyrosine, 2-hydroxy-3-naphthoic acid, protocatechuic aldehyde, pyrogallol, orcinol, salicylic acid, *p*-hydroxybenzoic acid, arbutin, euxanthic acid, baptisin, 2-hydroxyanthraquinone, β-methylglucoside, β-naphthol glucoside, catechin. The following have a moderately powerful effect. *m*- and *p*-Hydroxybenzaldehyde, β-resorcylic acid, gentisic acid, protocatechuic acid, vanillin, phlorhidzin, aloin, quercitrin, 4-hydroxyxanthone glucoside. Aesculin, ethyl gallate and tannin have a strong effect and the most active compound tested was 2-hydroxyxanthone glucoside; it is far more active than the isomeride. 2-Hydroxyxanthone glucoside also exerts a strong deepening and blueing effect on the colour of anthocyanins derived from cyanidin and the order of efficiency is cyanin $>$ chrysanthemin $>$ mecocyanin. The action on pelargonin is to darken the solution but not to make it more blue-red. Quercitrin blues oenin but does not increase the tinctorial intensity. We are indebted to Dr A. Robertson for pure specimens of his synthetic glucosides.

The authors wish to thank numerous friends for providing material and for assistance in the identification of specimens. They are deeply indebted to

Geheimrath Prof. R. Willstätter and to Prof. P. Karrer for specimens of anthocyanins. They have been greatly assisted by the generous help of Messrs Sutton and Sons of Reading.

REFERENCES.

Bradley, Robinson and Schwarzenbach (1930). *J. Chem. Soc.* 793.
Fleming (1929). *Scientific agriculture*, **10**, No. 4.
Haas and Hill (1929). An introduction to the chemistry of plant products, **1**, 340. (Longmans, Green & Co., London.)
Karrer and Widmer (1927). *Helv. Chim. Acta*, **10**, 67 and 729.
Kondo (1931). *J. Pharm. Soc. Japan*, **51**, 254.
Schudel (1918). Dissertation, Zürich.
Willstätter and Bolton (1916). *Liebig's Ann.* **412**, 138.
—— and Burdick (1916). *Liebig's Ann.* **412**, 217.
—— and Everest (1913). *Liebig's Ann.* **401**, 189.
—— and Mallison (1915). *Liebig's Ann.* **408**, 147.
—— and Mieg (1915). *Liebig's Ann.* **408**, 61.
—— and Weil (1916). *Liebig's Ann.* **412**, 231.
—— and Zollinger (1916). *Liebig's Ann.* **412**, 195.

CLXXXIII. AN ANTHOCYANIN OF *OXYCOCCUS MACROCARPUS* PERS.

BY KATHRYN E. GROVE AND ROBERT ROBINSON.

From the Dyson Perrins Laboratory, South Parks Road, Oxford.

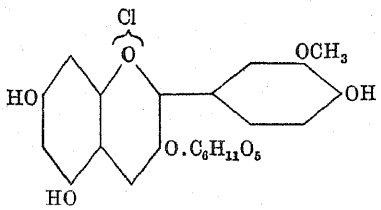
(*Received August 24th, 1931.*)

HAVING effected a synthesis of idaein chloride we wished to secure a supply of the natural pigment which Willstätter and Mallison [1915] isolated from the fruits of *Vaccinium vitis idaea* Linn. (this is the English cowberry, the true cranberry with smaller fruits being *V. oxycoccus*), but the latter not being in season we secured supplies of the much larger American cranberries in the hope that these might be a source of idaein.

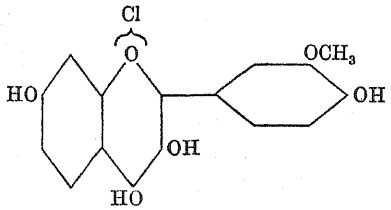
The first berries employed originated in Newfoundland and had a generally similar appearance to the second batch which came from the Cape Cod district.

The two were worked up separately, and the derived pigments were only combined when it was found that they had identical properties.

We have obtained the pure anthocyanin in small quantity only, but sufficient to establish its identity as a 3-*β-glucosidylpeonidin chloride* (I). On hydrolysis it yielded peonidin chloride (II), characterised by its analysis, habit of crystallisation and other properties, and by its colour reactions in buffered solutions.



(I)



(II)

The determination of the nature of the carbohydrate residue could not be made directly because of the paucity of material for investigation, but it happened that 3-β-glucosidylpeonidin chloride had been synthesised (Levy and Robinson) some time before the oxycoccicyanin was completely purified and direct comparison has shown that the two specimens are identical.

The most probable alternative to the glucosidic configuration is the galactosidic, and we therefore synthesised 3-β-galactosidylpeonidin chloride (paper submitted to the Chemical Society) and found that this anthocyanin closely resembled the glucoside. It differed, however, in its "distribution number" being less readily extracted by amyl alcohol from a solution in 0·5 % hydrochloric acid than is the glucosidic anthocyanin.

The new anthocyanin fills a gap in the series of monoglucosides, being a methoxycallistephin and a *des*methoxyoenin; we propose to designate it "oxycoccicyanin" following a precedent established by Nolan in naming "sambucicyanin." The alternatives are unsatisfactory, since "oxycoccin" suggests, perhaps, a derivative of carminic acid and "macrocarpin," referring simply to the size of the fruits, is not sufficiently specific.

Experimental.

(A) The cranberries from Newfoundland were rather smaller than those from Cape Cod; they were minced and submitted to great pressure in linen bags. We are indebted to Prof. J. C. Drummond for giving facilities and assistance at this stage. The skins were then extracted with acetic acid and the filtered extracts precipitated by the addition of ether. The syrupy material was again dissolved in acetic acid and precipitated by means of ether. The sticky red mass was then triturated with 2 % ethyl alcoholic hydrogen chloride and the solution filtered and precipitated by means of ether; this precipitate was dissolved in hot saturated aqueous picric acid and the solution kept in the ice-chest for several weeks. The crude picrate was extracted by means of boiling methyl alcohol and hydrogen chloride (to make a 1 % solution) introduced; the filtered solution was then mixed with an equal volume of ether. This precipitated impurities, including a cyanidin derivative (positive iron reaction); the filtrate from these was diluted with much ether and some light petroleum was also added. The intention had been to precipitate the chloride but, instead, the *picrate* crystallised on the sides of the vessels, which were kept for some weeks. The picrate could now be crystallised from hot ethyl alcoholic aqueous picric acid in red needles. After allowing ample time for complete separation the salt was collected, dissolved in hot methyl alcohol containing 2 % of hydrogen chloride and the chloride precipitated by means of ether. This gave a nearly pure amorphous specimen with a dark beetle-green lustre.

(B) The peelings of Cape Cod cranberries (60 lb.) were allowed to stand in 2 % methyl alcoholic hydrochloric acid for 2 to 3 weeks, using the counter flow method with the solvent. The solution was then filtered, the solvent being carefully pressed from the peelings, which were then washed with fresh solvent, the filtrate being added to the original solution. The colour was not entirely removed, but the skins were very light coloured after this treatment.

The alcoholic solution (*ca.* 11,000 cc.) was then divided into portions of 500 cc. and each portion was treated as follows: dry picric acid (10 g.) was added and the mixture was warmed to 40° and then poured into a large evaporating dish. Concentrated ammonium hydroxide (20 cc., the amount calculated almost to neutralise the acid present) was added and the mixture was thoroughly stirred and then allowed to stand in a draught chamber for 2 weeks; at the end of that time there was a crystalline residue covered with a sticky gum. The residues were collected, washed with saturated aqueous picric acid (1000 cc.), and quantities of ether, and dried (200 g.) (X).

This product was divided into 25 g. portions and each portion was boiled with absolute methyl alcohol to remove the pigment present. The combined extracts (*ca.* 1500 cc.) were filtered and dry ether (3 vols.) was added to the filtrate. A heavy precipitate formed on the addition of the ether, and this was filtered off and dried; very little anthocyanin was found in this material.

The filtrate was diluted with an equal volume of light petroleum (B.P. 40–60°) and the anthocyanin extracted by means of 1 % hydrochloric acid (500 cc.). The solution was then evaporated in a vacuum at the ordinary temperature and the picrate slowly separated. This was collected, dried and dissolved in the minimum of 3 % ethyl alcoholic hydrogen chloride; the solution was kept, filtered and the chloride precipitated by the addition of ether; the last process was repeated. The anthocyanin at this stage was compared with the product of process (A) from the Newfoundland berries and found to be entirely identical in alkali-colour reactions and in distribution number, using amyl alcohol with aqueous hydrochloric acid and ethyl acetate with aqueous picric acid. The specimens were therefore combined (see below).

The pigment was completely extracted from a typical berry with formic acid (1·5 cc.), the solution was filtered and diluted to 50 cc. with absolute methyl alcohol, and the amount of pigment determined colorimetrically by comparison with a solution of 2·0 mg. of idaein in methyl alcohol (50 cc.). The two solutions were approximately identical in colour. A pound of berries contains approximately 500 berries, *i.e.* 1·0 g. of pigment.

A determination of the relative amounts of anthocyanidin, monoglucoside, and diglucoside present in the mother-liquor from the original picrate precipitate (X) was made as follows. The anthocyanidin was extracted by means of ether saturated with picric acid, the monoglucoside by carvone-picric acid, and the diglucoside by amyl alcohol-acetophenone (2 : 1 vol.) picric acid solution. In each case the solution of pigment was shaken with the extraction medium until no more colour was removed, the combined extracts were then diluted with ether (light petroleum in the case of anthocyanidin) and shaken out with 0·5 % hydrochloric acid (50 cc.). This aqueous solution was freed from picric acid by extraction with ether and then compared colorimetrically with cyanidin, 3-β-galactosidylpeonidin chloride or mecocyanin chloride. The solution was found to contain 0·03 mg. anthocyanidin, 0·21 mg. monoglucoside, and 0·07 mg. diglucoside per cc. This procedure is due to (Willstätter and) Schudel [1918].

Examination of the anthocyanin. The crude chloride (0·8 g.) was dissolved in warm 5 % ethyl alcoholic hydrogen chloride, but crystallisation did not occur on cooling, and the solution was kept in a vacuum in a vessel containing ether. The alcohol was gradually diluted with ether and a poor type of crystallisation ensued. The indefinitely crystalline crust was then triturated with 5 % alcoholic hydrogen chloride and, although a large loss resulted, an insoluble material was obtained which could be crystallised by dissolution in 0·5 % hydrochloric acid and addition of 5 % ethyl alcoholic hydrogen chloride, after

which the solution was kept in the ice-chest. A recrystallisation in the same way furnished rectangular, prismatic needles, deep-red by transmitted light under the microscope and almost black in mass. (Found in air-dried material: C, 47·9; H, 5·4; Cl, 6·5; loss at 105° in a high vacuum over phosphoric anhydride, 7·4, 7·9. $C_{22}H_{23}O_{11}Cl$, $3H_2O$ requires C, 47·8; H, 5·3; Cl, 6·4; $2H_2O$, 6·5. Found in the material dried at 105°: C, 51·0; H, 5·2; Cl, 6·6; MeO, 6·1. $C_{22}H_{23}O_{11}Cl$, H_2O requires C, 51·2; H, 4·8; Cl, 6·8; MeO, 6·0 %.)

The synthetic 3-glucosidylpeonidin chloride contains $2H_2O$, both molecules being evolved at 110° in a high vacuum. Little importance is, however, attached to this divergence, since it has been the repeated experience of workers in the anthocyanin field that the degree of hydration of the salts can be greatly affected by small concentrations of foreign substances.

Hydrolysis to the anthocyanidin. The last two alcoholic mother-liquors contained the major part of the pigment, and the alcohol was removed by distillation; the residue was then heated on the steam-bath until a test with amyl alcohol showed that the anthocyanin had all been hydrolysed. Care was taken not to heat any longer than was necessary. The anthocyanidin was converted into picrate and this was crystallised from methyl alcohol and then transformed into the chloride by means of methyl alcoholic hydrogen chloride and ether. The precipitated chloride was well dried and crystallised by dissolution in hot ethyl alcohol and addition of one-third of the volume of 12 % hydrochloric acid. It separated in prisms having a green reflex and appearing orange-brown by transmitted light under the microscope. (Found: C, 52·5; H, 4·7; loss at 105° in a high vacuum, 7·4. $C_{16}H_{13}O_6Cl$, 1·5 H_2O requires C, 52·8; H, 4·4; 1·5 H_2O, 7·4. Found in anhydrous material: C, 57·2; H, 4·2; MeO, 9·4. $C_{16}H_{13}O_6Cl$ requires C, 57·1; H, 3·9; 1MeO, 9·2.)

Peonidin chloride synthesised by Murakami and Robinson [1928] was crystallised in the same way and with the same results (1·5 H_2O), but this is the first time that the prism-crystals have been obtained with peonidin from natural sources. The very small amount of the pure peonidin chloride that remained was employed for a study of the colour reactions in buffered solutions of graded p_H. There was only sufficient for twelve tests and the solutions numbered (2)–(13), inclusive in the memoir of Robertson and Robinson [1929], were employed; the results with these twelve solutions agreed completely with the description there given for peonidin.

Properties of oxycoccicyanin chloride and comparisons with 3-β-glucosidylpeonidin chloride. The anthocyanin is rather readily soluble in 0·5 % hydrochloric acid; much less readily soluble in 5 % hydrochloric acid to solutions which become very much more blue-red on dilution with alcohol. Its solution in aqueous sodium carbonate is rich violet in colour and there is no change on the addition of sodium hydroxide, except that the colour soon fades becoming greenish yellow.

The *absorption* of light in the visible region was identical with that of a specimen of 3-β-glucosidylpeonidin (see Fig. 1). The slight divergence from

3-β-galactosidylpeonidin is not significant and probably means that the galactoside was exceptionally pure.

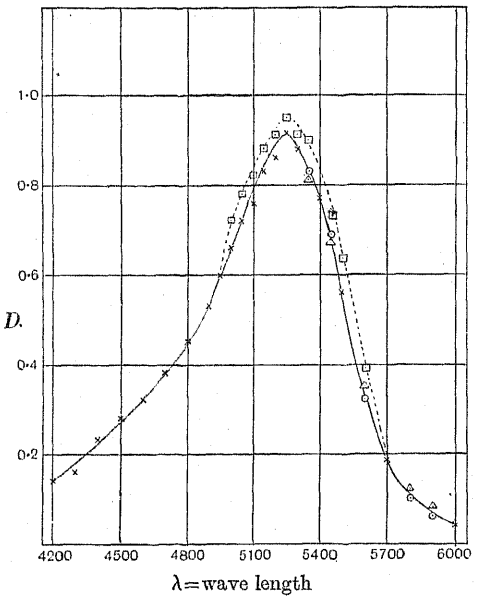


Fig. 1. ⊙ 3-β-Glucosidylpeonidin chloride (natural).
△ 3-β-Glucosidylpeonidin chloride (synthetic).
⊡ 3-β-Galactosidylpeonidin chloride.
× Coincident points.

The *distribution number* was determined using 0·5 % hydrochloric acid (50 cc.) and amyl alcohol (50 cc.). Using 2·432 mg. of the anthocyanin, 26·6 % passed into the alcoholic layer; a second shaking extracted 28·5 % of the material remaining in the aqueous solution. Using 4·907 mg. the corresponding figures were 21·4 % and 22·3 %. These figures did not agree as sharply as we anticipated either with the synthetic glucoside or synthetic galactoside.

However, we had previously tested the crude anthocyanin against glucosidylpeonidin and found identity, so that we were inclined to suspect that the conditions were in some way not quite standard. Using exactly the same conditions, that is the same actual samples of 0·5 % hydrochloric acid and amyl alcohol, we found that with synthetic 3-β-glucosidylpeonidin chloride (5·017 mg.) the distribution numbers for the first and second shakings were 21·9 and 22·6 respectively.

Colour reactions in buffered solutions. The solutions employed were those specified by Robertson and Robinson [1929], and oxycoccicyanin chloride, 3-β-glucosidylpeonidin chloride and 3-β-galactosidylpeonidin chloride gave identical results, except that a few notes on very slight and probably non-significant divergences of the galactoside were made. (2) salmon pink, rapidly fading; (3) similar, fading more rapidly; (4) a little bluer, also fading;

(5) cherry red, much more stable; (6) bluer, more intense, more stable; (7) similar but brownish tinge; (8) a shade bluer; (9) similar, dull red-violet; (11) sharp change to deep violet; (13) the same; (450 cc.), the same but rapidly fading to smoky colour and then yellow. After 1 hour: (2), (3), (4) and (5) formed a decreasing faded series; (6) faded, but intensity between (2) and (3), the galactoside a little the more stable; (7), (8) faded brown-violet; (9) bluer and more intense; (11), (13) violet, little changed; (450 cc.), yellow. Hydrochloric acid added to (4) and (5) restored the colour quantitatively. After 24 hours the solutions in the three series were identical: (2) pink; (3) nearly colourless; (6) colourless; (7), (8), (9) gradual increase to smoky violet; (11), (13) good violet; (450 cc.) yellow.

The identical colour reactions of chrysanthemin chloride and idaein chloride (synthetic specimens) have also been observed by the standard method. (1) salmon red; (2) a little bluer; (3) a little bluer, fading most rapid here; (4) cherry red of greater tinctorial intensity; (5) deeper, fades more slowly; (6) still deeper; (7) duller and browner; (8) still browner; (9) brown-red; (10) the same; (11) the same; (12) the same; in the series (9)–(12) we thought idaein had a little the greater intensity; (13) brown-violet; (14) reddish violet; (15) violet, (13)–(15) lose the blue tinge on keeping; (16) much bluer violet; (17) pure blue fading to greenish blue and then yellow. After 24 hours the 2 series were identical: (1) salmon red; (2) weak bluish red with small precipitate; (3), (4) nearly colourless with violet precipitate; (5) weak violet and precipitate; (6) violet, no precipitate; (7), (8), (9), (10), (11) increasing series to violet-red; (12), (13) a little weaker colour than (11); (14) much weaker violet-red; (15), (16) weak yellow; (17) more intense yellow.

The authors wish to thank the National Research Council (U.S.A.) for a Fellowship awarded to one of them.

Summary.

The fruits of *Oxycoccus macrocarpus* Pers. have been found to contain a pigment which has been isolated as the chloride and identified as a 3-glucoside of peonidin by analysis and comparison with a synthetic specimen.

REFERENCES.

Murakami and Robinson (1928). *J. Chem. Soc.* 1537.
Robertson and Robinson (1929). *Biochem. J.* **23**, 35.
Schudel (1918). Dissertation, Zürich.
Willstätter and Mallison (1915). *Liebig's Ann.* **408**, 15.

CLXXXIV. THE ERGOTHIONEINE CONTENT OF THE BLOOD IN HEALTH AND DISEASE.

By HAROLD BERNARD SALT.

From the Biochemical Department of the Children's Hospital, Birmingham.

(*Received August 27th, 1931.*)

It is now definitely established that ergothioneine is present in the corpuscles of human blood and of certain animal bloods. Hunter and Eagles [1925] isolated the compound from pig's blood and later [1927] referred to its isolation from children's blood. Benedict, Newton and Behre [1926] also isolated the substance from both pig's and human blood. Hunter [1928] has briefly surveyed the history of ergothioneine and has pointed out the importance of two delicate colour tests to which the substance responds, the development of a blue colour with the uric acid reagents and a new reaction developed by Hunter from the Ehrlich diazo-reaction. Methods for the estimation of ergothioneine based upon the colour reaction with the uric acid reagents were used by Benedict *et al.* [1926, 1929, 1, 2], and by Rockwood, Turner and Pfiffner [1929]. In the earlier publication of Benedict, the following quantitative data were given:

Normal human blood	14–15 mg. ergothioneine per 100 cc.
Diabetic ,, ,,	20–27 mg. ,, ,, ,,
Nephritic ,, ,,	Normal or low values.

However, in Benedict's later publication, the above figures were stated to be too high. A new normal average for human blood was given as 7·5 mg. per 100 cc. whole blood (range 4·2–15·0), and apparently no further study of pathological cases was made.

Rockwood *et al.* [1929] show values for the ergothioneine content of various pathological bloods "in terms of uric acid." The factor of conversion of "ergothioneine in terms of uric acid" to actual ergothioneine was not determined or used by them for the particular method of estimation employed. It thus appeared that more reliable information concerning the ergothioneine content of pathological blood would be desirable. The work fell into two sections: firstly, the development of a reliable technique for the estimation of ergothioneine, which could be also combined with the blood-uric acid estimation, and secondly, the application of this to a series of blood specimens.

Discussion of certain factors in connection with the estimation of ergothioneine.

Benedict *et al.* [1926] found that, in the preparation of protein-free blood filtrates, some tungstic acid reagents caused a loss of all or much of the ergothioneine. This was confirmed by Eagles and Vars [1928]. As a remedy, Benedict

and Newton [1929, 1] adopted a molybdic acid reagent and later [1929, 2] a tungstomolybdic acid reagent for the removal of blood-proteins. Each of these reagents was stated to cause no precipitation of ergothioneine.

In order to choose a suitable blood-protein precipitant, a series of thirty blood samples (mostly pathological) has been used to determine which of the above three methods of protein removal yielded blood-filtrates with the greatest content of ergothioneine. As both uric acid and ergothioneine contribute to the colour developed in the "direct" uric acid estimation, "direct" uric acid values were compared for this purpose. It was found that using reagents prepared from the samples of chemicals stocked in this laboratory, the tungstomolybdic method caused least loss of ergothioneine, and the molybdic method most loss. The tungstic method was found to be nearly as satisfactory in this respect as the tungstomolybdic method (Table I).

Table I.

	"Direct" uric acid, mg. per 100 cc.		
24 samples of human blood	Tungstic	Molybdic	Tungstomolybdic
Maximum	8·1	—	8·6
Minimum	1·6	1·1	1·6
Mean of 24	3·70	—*	3·75
6 samples of pig's blood			
Maximum	1·5	1·8	2·4
Minimum	0·9	0·6	1·2
Mean	1·17	1·03	1·54

* 14 samples only, estimated by the molybdic method; all the figures were definitely low.

This indicated that the use of tungstomolybdic acid as a protein precipitant would be most desirable in blood-ergothioneine determinations. A support for this conclusion was established by the finding that ergothioneine added to blood could be quantitatively "recovered" from tungstomolybdic filtrates. The ergothioneine used was very kindly supplied by Dr G. Hunter.

Folin claimed [1930] that the whole of the ergothioneine of normal blood is retained within the corpuscles when the unlaked blood extract is prepared. That this is also true for pathological bloods has been shown by an examination of twenty-five blood specimens. In the same experiments it was also shown that the centrifugate obtained in the preparation of unlaked extracts, which contains the whole of the blood-ergothioneine, could be used for the estimation of ergothioneine. The actual method, in an improved form, is described later in this paper.

Table II.

	Uric acid, or "ergothioneine in terms of uric acid," mg. per 100 cc.		
	Unlaked extract		
25 human blood specimens	Uric acid	"Ergothioneine"	Centrifugate, "Ergothioneine"
Maximum	13·3	< 0·2*	1·0
Minimum	1·3	—	0·2

* By the method used for this series, amounts of less than 0·2 mg. per 100 cc. blood could not be estimated. The actual values probably approached zero.

The method of differential determination of uric acid and ergothioneine.

The following reagents were used:

(1) Sulphate-tungstate solution, containing 34 g. crystallised sodium sulphate and 6 g. sodium tungstate per litre of solution.

(2) *N*/3 sulphuric acid.

(3) Tungstomolybdic reagent of Benedict and Newton [1929, 2].

(4) 0·62 *N* sulphuric acid.

(5) Acid silver lactate reagent prepared according to Folin [1922]. The solution was always filtered immediately before use.

(6) 10 % NaCl in 0·1 *N* HCl.

(7) Cyanide-urea reagent of Folin [1930].

(8) Folin-Marenzi uric acid reagent [1929].

(9) Standard uric acid solution of Folin [1930].

4 cc. oxalated non-haemolysed whole blood were added to 32 cc. sulphate-tungstate solution. After standing with occasional gentle rotatory shaking for 15 minutes, 4 cc. *N*/3 sulphuric acid were added slowly and with shaking, and the whole mixture was centrifuged as completely as possible. The supernatant liquid was poured off completely and used for the determination of uric acid by the method of Folin [1930].

The centrifugate obtained was transferred quantitatively with small amounts of water and repeated shaking to a long narrow test-tube graduated at 32 cc. about half-way up. The volume was made up with water practically to this mark and the froth removed by means of a trace of capryl alcohol. The volume was then made up to 32 cc. and the contents of the tube were mixed by closing with a bung and inverting several times, avoiding further frothing. This process was found completely to lake the corpuscles. 4 cc. tungstomolybdic reagent were then added and the contents of the tube mixed again, this effecting re-solution of much of the precipitated plasma-proteins, and causing a colour change from opaque red-brown to a clear deep red. The whole mixture was then poured completely into a wide-necked flask, adequate time being allowed for drainage. 4 cc. 0·62 *N* sulphuric acid were then added slowly and with shaking to precipitate the whole of the protein, after which the mixture, occupying a total of 40 cc., was filtered through a 9 cm. No. 40 Whatman paper.

20 cc. filtrate were almost always available and were transferred to a round-bottomed cylindrical centrifuge tube. If 20 cc. were not available, some other aliquot portion, say 18 cc., was used and a correction applied in the calculation. To the filtrate, 7 cc. acid silver lactate solution were then added, and, after standing for a few minutes in the dark, the precipitate was centrifuged. The supernatant liquid was discarded and 1 cc. acid sodium chloride added to the precipitate, which was then thoroughly extracted by triturating with a rounded glass rod. The rod was washed down with about 4 cc. water and the precipitate again centrifuged. The supernatant liquid, containing any uric acid

present, was discarded and the extraction with sodium chloride and water repeated. Finally, 5 cc. cyanide-urea reagent were added to the precipitate which was dissolved by stirring, and the solution was washed quantitatively into a large hard glass test-tube graduated at 14 cc. and water added to this mark.

As a standard, 2 cc. dilute "blood-uric acid" standard solution (containing 0·008 mg. uric acid) were transferred to a similar test-tube, and 5 cc. cyanide-urea reagent and 7 cc. water added.

To both standard and unknown, 1 cc. uric acid reagent was then added and mixed. After allowing the tubes to stand for 4 minutes at room temperature, they were heated in a boiling water-bath for 2 minutes and then rapidly cooled in running water. The colours were compared in the colorimeter, without further dilution of the liquid. The unknown solution was set at 20 mm. and the standard colour matched against it. The calculation was made from a graph plotted for the particular reagents used. This was found necessary, mainly on account of the variable "blank" colours yielded by different samples of sodium cyanide. For the estimations here recorded, the following graph was obtained.

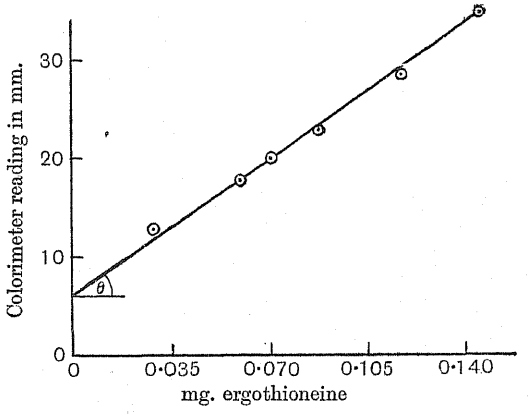


Fig. 1.

The graph is produced back to cut the ordinate at a point representing 6·0 mm., *i.e.* the equivalent of the "blank" colour. The slope of the line, $\tan\theta = 0{\cdot}7$.

If c be the unknown concentration of ergothioneine in a test portion,

y be the reading of the standard colour, matched against the unknown set at 20 mm.,

a be the value in mm. represented by the "blank" colour (constant),

then
$$c = \frac{y - a}{\tan\theta}.$$

This actually reduces to
$$c = \frac{y - 6}{200} \text{ mg.}$$

In the estimation of blood-ergothioneine, the test portion contains the ergothioneine of 2 cc. blood, if 20 cc. filtrate are used, hence

$$\text{mg. ergothioneine per 100 cc. whole blood} = \frac{y-6}{200} \times \frac{100}{2} = \frac{y-6}{4}.$$

The standard colour was found to be stable for only about 1 hour, and hence was conveniently prepared for each ergothioneine estimation. The use of uric acid as a standard was found to be quite suitable, while various dyes were unsatisfactory. The use of pure ergothioneine would necessitate a demand for this substance which cannot as yet be met.

Glutathione and cysteine present in blood in normal amounts exert no interfering effect upon the accuracy of ergothioneine determinations by this method. This has been shown by experiments in which ergothioneine was estimated in blood, with and without the addition of these substances. Finally, the method was found to yield satisfactory duplicate figures.

The findings for normal and pathological blood.

As the ergothioneine of the blood is confined to the corpuscles and as the percentage volume of corpuscles varies, particularly in pathological cases, the corpuscle volume was determined for each blood sample. Uric acid and ergothioneine were estimated by the method described above, the figures being obtained as mg. per 100 cc. whole blood. The ergothioneine was then calculated from the corpuscle volume, as mg. per 100 cc. corpuscles. The results of 60 estimations are shown in Table III. For each group, the figures are arranged in order of increasing ergothioneine content. Blood-sugar values are included for the diabetic bloods and urea values for most of the other pathological samples.

The most striking feature about the results is the large range of ergothioneine values in all the human blood groups studied. The normal range (3–12 mg. per 100 cc. corpuscles) agrees well with that given by Hunter [1928], using an entirely different method of estimation. This range is such as to obscure any variation from the normal due to pathological conditions, and to yield a mean figure of little significance. There is no evidence of any constant variation of the blood-ergothioneine content with sex or with age. Eagles and Vars [1928] have shown that the dietary history of pigs determines the blood-ergothioneine content. However, it is considered that normal human subjects, such as were studied, would be receiving a mixed and balanced diet, without any undue amount of maize protein or other material rich in ergothioneine-producing substances.

In the diabetic series the ergothioneine figures certainly tend to be slightly higher than the normal, but again the range of values is so great as to make this variation of questionable significance. There is no correlation between the ergothioneine and sugar values; neither was any variation in the ergothioneine value related to any stage of the disease or to the treatment. However, this point merits more detailed study.

Table III.

(*a*) *Normal blood.*

Specimen	Age	Sex	% volume of corpuscles	Uric acid, mg. per 100 cc. blood	Ergothioneine, mg. per 100 cc. corpuscles
B.	Adult	F.	47	2·80	3·05
Mixed	,,	—	40	2·92	5·60
C.	,,	M.	49	2·40	6·70
M.	,,	M.	46	3·67	6·85
A.	,,	F.	45	2·75	11·60
D.	,,	F.	51	3·64	11·60
L.L.	,,	F.	45	3·15	12·00
S.S.	11	M.	42	2·93	3·55
B.L.	10	M.	43	2·65	4·05
D.B.	3	M.	37	1·73	6·90
G.H.	4	M.	39	3·32	8·20
	Mean of normal samples		... ...	2·85	7·28

(*b*) *Diabetic blood.*

						Sugar, mg. per 100 cc. blood
J.G.	65	F.	46	2·95	6·95	263
E.J.	25	M.	41	2·39	8·15	220
W.	Adult	F.	44	2·38	8·30	180
V.H.	26	F.	45	2·25	8·55	340
C.	Adult	F.	46	1·50	8·80	136
E.B.	23	F.	51	2·52	8·90	250
A.B.	29	F.	45	2·33	9·00	347
A.K.	70	F.	50	3·17	9·30	144
E.H.	61	F.	48	3·39	10·10	208
F.C.	25	M.	53	1·51	10·60	76
W.M. (*a*)	39	M.	41	3·20	11·10	109
N.S.	15	M.	42	2·92	14·05	260
W.M. (*b*)	39	M.	33	3·33	15·15	111
P.L.	10	M.	40	2·94	7·90	170
G.H.	8	M.	41	1·86	9·50	98
R.W. (*a*)	Child	M.	31	5·13	11·95	200
R.W. (*b*)	,,	M.	28	5·16	14·65	266
	Mean of diabetic samples		... ...	2·85	10·17	

(*c*) *Nephritic blood.*

						Urea, mg. per 100 cc. blood
M.M. (*a*)	12	M.	29	1·72	4·85	129
B.R.	8	F.	49	2·13	4·90	81
M.M. (*b*)	12	M.	44	1·75	5·25	42
B.H.	7	M.	42	2·63	5·70	80
D.C.	6	M.	56	5·06	5·70	65
I.W.	10	F.	36	4·19	5·85	45
B.G.	12	F.	40	2·94	6·00	59
T.H. (*a*)	12	M.	33	2·92	6·35	54
O.K.	6	F.	29	1·98	7·40	39
T.H. (*b*)	12	M.	46	2·20	7·80	47
G.T.	6	F.	33	2·29	8·20	22
C.W.	5	F.	42	1·82	8·55	42
K.C.	2	M.	21	3·50	10·00	84
M.R.	10	F.	32	2·67	14·55	35
	Mean of nephritic samples		... ...	—	7·78	—

Table III (*continued*).

(*d*) *Various pathological bloods.*

Specimen	Age	Sex	% volume of corpuscles	Uric acid, mg. per 100 cc. blood	Ergothioneine, mg. per 100 cc. corpuscles	Urea, mg. per 100 cc. blood
Chorea	10	F.	44	0·97	4·00	33
Prostatitis	55	M.	44	2·59	4·00	40
?	37	M.	46	3·64	4·55	40
Chorea	12	M.	37	1·51	5·00	—
?	57	F.	40	3·69	5·75	67
?	47	M.	25	2·40	6·00	—
Coeliac rickets	7	F.	37	3·43	6·75	76
?	Adult	M.	50	6·56	8·00	—
? Renal tumour	11	M.	48	3·08	9·15	—
Pyelitis	9	F.	46	1·78	9·80	48
Rheumatism	8	M.	43	1·65	10·25	40
Pulm. T.B.	10	F.	45	2·32	13·75	28
Hemiplegia	67	F.	50	3·85	14·70	—
Mean of miscellaneous pathological samples ...				—	7·82	—

(*e*) *Pig's blood.*

1	—	—	43	0·36	15·60	—
2	—	—	50	0·35	20·00	—
3	—	—	45	0·37	21·10	—
4	—	—	36	0·32	22·80	—
5	—	—	55	0·34	24·20	—
	Mean ...	...	... 46	0·35	20·74	—

In the nephritic series the ergothioneine values are normal and do not vary with the retention of nitrogenous excretory products as evidenced by the uric acid and urea figures. A similar large range of values is shown for thirteen miscellaneous pathological blood samples. On the other hand, four out of five specimens of pig's blood, obtained from the abattoir, yielded ergothioneine values substantially higher than those of human blood.

The uric acid figures are of interest as they were obtained by the new method of Folin [1930], for which normal figures have not yet been fully established. In other experiments with both normal and pathological human blood the author has found that normal figures by this new method are consistently slightly lower than those obtained by the older "direct" technique of Folin [1923] for the same blood.

Summary.

A new method for the differential determination of uric acid and ergothioneine in blood is described. Figures are recorded for the uric acid and ergothioneine contents of normal and certain pathological bloods.

The expenses of this research have been partially covered by a grant made by the Medical Research Council to Prof. Parsons. I am indebted to him and to other members of the Hospital Staff for their interest and co-operation.

REFERENCES.

Benedict and Newton (1929, 1). *J. Biol. Chem.* **82**, 5, 11.
—— —— (1929, 2). *J. Biol. Chem.* **83**, 357, 361.
—— —— and Behre (1926). *J. Biol. Chem.* **67**, 267.
Eagles and Vars (1928). *J. Biol. Chem.* **80**, 615.
Folin (1922). *J. Biol. Chem.* **54**, 153.
—— (1923). Laboratory manual of biological chemistry. (Appleton, London and New York.)
—— (1930). *J. Biol. Chem.* **86**, 173, 179.
—— and Marenzi (1929). *J. Biol. Chem.* **83**, 109.
Hunter (1928). *Biochem. J.* **22**, 4.
—— and Eagles (1925). *J. Biol. Chem.* **65**, 623.
—— —— (1927). *J. Biol. Chem.* **72**, 123.
Rockwood, Turner and Pfiffner (1929). *J. Biol. Chem.* **83**, 289.

CLXXXV. THE COLORIMETRIC DETERMINATION OF URIC ACID IN URINE.

By HAROLD BERNARD SALT.

From the Biochemical Department of the Children's Hospital, Birmingham.

THE colorimetric method of estimating uric acid in urine has not received attention in the same way as the method for estimating uric acid in blood. Although one of the difficulties of the blood-uric acid determination, namely the development of turbidity in the final solution used for colorimetric comparison, has been entirely overcome, the urine-uric acid method has not been studied, so far as is known, with a view to overcoming this difficulty of cloudy solutions. The Folin method of estimating uric acid in urine [1923] invariably gives turbid final solutions. It has been found always necessary to centrifuge before making the colour comparison. This procedure is satisfactory, except that frequently the turbidity is not completely removed, and even where clear solutions are obtained a fresh clouding often develops while the comparison is being made. Accordingly, the effect of substituting the improved uric acid reagents in Folin's method for urine-uric acid has been investigated and the conditions of reaction modified so that the final solutions are perfectly clear.

Briefly, Folin's method depends upon the reduction of phospho-18-tungstic acid by uric acid in the presence of a large excess of sodium carbonate. Sodium cyanide is also present and the reaction is allowed to proceed at room temperature.

EXPERIMENTAL.

The following reagents were employed.

(1) Uric acid reagent of Folin and Marenzi [1929].

(2) Uric acid standard solution of Folin [1930] containing 1 mg. uric acid per cc. This was diluted ten times with water alone, to prepare the working standard when required.

(3) Cyanide-urea reagent of Folin [1930].

(4) 15 % aqueous sodium cyanide.

(5) Acid silver lactate reagent prepared by the method of Folin [1922]. The solution was always filtered immediately before use.

(6) Sodium carbonate solution containing 20 g. anhydrous sodium carbonate per 100 cc. solution. This is referred to as "20 % sodium carbonate." Suitable dilutions were made to prepare other strengths, *e.g.* "10 % sodium carbonate" (1 : 1 dilution).

The influence of the concentration of sodium carbonate upon colour development and formation of turbidity.

5 cc. portions of dilute standard uric acid solution (each containing 0·5 mg. uric acid) were transferred to each of a series of eleven 100 cc. graduated flasks. To each was added 20 cc. sodium carbonate solution of different concentrations as indicated below. 2 cc. 15 % sodium cyanide were then added to each flask, followed by 5 cc. uric acid reagent. The contents of the flasks were mixed and, after standing for 5 minutes at room temperature, diluted to 100 cc. with water. The turbid solutions were then quickly centrifuged and the colours compared in the colorimeter, using the first solution as a standard set at 20 mm.

Table I.

% concentration of sodium carbonate used	20·0	17·5	15·0	12·5	10·0	7·5	5·0	2·5	2·0	1·5	1·0
Colorimeter reading in mm.	20·0	19·0	18·3	16·6	15·5	14·7	12·8	11·5	11·6	19·9	44·7
Precipitate formation	++	++	++	+	+	±	–	–	–	–	–

It is seen that a reduction in the concentration of sodium carbonate used from 20 % to 2·5 % causes a steady increase in the amount of colour developed. However, the formation of precipitate is only just avoided at the 5 % concentration.

The experiment was then repeated exactly, except that 5 cc. cyanide-urea reagent were substituted for the 2 cc. 15 % aqueous sodium cyanide. The results of this series are below.

Table II.

% concentration of sodium carbonate used	20·0	17·5	15·0	12·5	10·0	7·5	5·0	2·5	2·0	1·5	1·0
Colorimeter reading in mm.	20·0	18·3	17·4	16·3	15·5	14·3	12·6	10·6	10·9	12·7	26·7
Precipitate formation	Trace	Trace	Trace	Trace	–	–	–	–	–	–	–

Again, the colour developed was progressively greater as the concentration of sodium carbonate used was decreased from 20 % to 2·5 %. The formation of precipitate was very slight in the first four solutions, and entirely avoided in the 10 % concentration and below.

Application of the new technique to urine.

As a result, it was proposed at first to use 20 cc. 2·5 % sodium carbonate and 5 cc. cyanide-urea reagent in the uric acid determination as, under these conditions, no precipitate formed in the final solution, and colour development was at a maximum. This modified technique was applied to urine and the results compared with those obtained by Folin's original method.

Two portions of 1 cc. urine were transferred to each of two centrifuge tubes and 3 cc. water followed by 3 cc. acid silver lactate added to each. After mixing and standing in the dark for a few minutes, the tubes were centrifuged and

the supernatant liquid was discarded. One precipitate was dissolved in 2 cc. 15 % sodium cyanide and transferred with 5 cc. water to a graduated 100 cc. flask. The colour was then developed, using 20 cc. 20 % sodium carbonate, and matched against a standard prepared from 5 cc. dilute uric acid standard solution, after centrifuging to remove the precipitate. The result was calculated in grams uric acid per 100 cc. urine. The second silver precipitate was dissolved in 5 cc. cyanide-urea reagent and treated in a similar way except that 2·5 % sodium carbonate was used. The colour comparison was made with an appropriate standard. The type of result obtained is shown in Table III.

Table III.

Specimen	Folin method (15 % NaCN, 20 % sod. carb.)	New technique (Cyanide-urea, 2·5 % sod. carb.)
1	0·064	0·058
2	0·053	0·045
3	0·074	0·061

It is seen that the new method yielded results much below those of Folin's original method, this being apparently due to the reduction in the concentration of sodium carbonate used. Hence, 10 % sodium carbonate was chosen as a possible suitable concentration of alkali. It had already been found that this solution, in combination with the cyanide-urea reagent, yielded the desired colours without turbidity, but at the same time maintained the reaction mixture very strongly alkaline.

Using a number of urine samples, a comparison was made as above between the original Folin method and the new method, using cyanide-urea and 10 % sodium carbonate. Typical results are shown below.

Table IV.

Specimen	Folin's method (15 % NaCN, 20 % sod. carb.)	New technique (Cyanide-urea, 10 % sod. carb.)
1	0·044	0·043
2	0·062	0·063
3	0·051	0·053
4	0·119	0·123
5	0·068	0·067
6	0·051	0·052
7	0·044	0·045
8	0·033	0·033
9	0·074	0·076
10	0·060	0·060
Average	0·0606	0·0615

Agreement between the two methods is here seen to be quite close. In no case was any turbidity produced except when using urines containing less than 0·025 % uric acid. In such a case it is considered that the uric acid reagent is in great excess of the amount needed to react with the uric acid, and this excess is decomposed by the alkali, giving a turbidity. This difficulty was easily avoided by using more than 1 cc. urine in the first treatment with acid silver lactate.

Within limits the new technique was found to give a good degree of proportionality between the uric acid content and colour intensity. This was shown by estimating the uric acid by the new technique in different quantities of the same urine, calculating each estimation to g. per 100 cc.

Table V.

cc. urine taken	1·0	1·5	2·0
Colorimeter reading when standard set at 15 mm.	27·4	17·9	13·8
% uric acid in the urine	0·027	0·028	0·027

It is recommended that uric acid be estimated in urine by the following method.

Transfer 1 cc. urine (or more if the uric acid content is low) to a centrifuge-tube. Add 3 cc. water and 3 cc. acid silver lactate solution. Mix, stand in the dark for a few minutes, then centrifuge. Discard the supernatant liquid. Dissolve the precipitate in 5 cc. cyanide-urea reagent and transfer quantitatively to a 100 cc. graduated flask, using successively two portions of 10 cc. 10 % sodium carbonate solution and finally 5 cc. water.

Prepare a standard by transferring to a 100 cc. graduated flask 5 cc. uric acid standard solution (containing 0·5 mg. uric acid), 5 cc. cyanide-urea reagent and 20 cc. 10 % sodium carbonate solution.

To each flask add 5 cc. uric acid reagent, mix and allow to stand 5 minutes. Dilute to volume, mix and compare in the colorimeter.

SUMMARY.

A modification of Folin's method of estimation of uric acid in urine is described. It is easily carried out in routine practice, and yields results strictly comparable with those obtained by Folin's original technique. It is free from the objection of turbidity in the final colours.

REFERENCES.

Folin (1922). *J. Biol. Chem.* **54**, 153.
—— (1923). Laboratory manual of biological chemistry, p. 141. (Appleton, London and New York.)
—— (1930). *J. Biol. Chem.* **86**, 179.
—— and Marenzi (1929). *J. Biol. Chem.* **83**, 109.

CLXXXVI. BLOOD-PHOSPHATASES.

By JEAN ROCHE.

From the Biochemical Department, Lister Institute, London.

(*Received August 28th, 1931.*)

Previous workers [Martland, Hansman and Robison, 1924; Demuth, 1925; Martland and Robison, 1926; Kay, 1929, 1930, 1 and 2] have shown that the red corpuscles and plasma of mammalian blood each contain a phosphatase, but that these enzymes differ in certain characteristics notably in their optimum p_H. Kay [1930, 1] also noted the occurrence of a phosphatase in the leucocytes but made no further study of its properties.

In the present work these phosphatases of the red and white cells and of plasma have been studied and compared with those of other tissues, especially of bone and kidney. It is probable that the phosphatase of the red cells participates in the mechanism of glycolysis in blood [Roche and Roche, 1929] and it was therefore considered that further knowledge of the hydrolytic and synthetic powers of this enzyme was desirable and might help towards understanding the glycolytic process.

Experimental.

The cells were separated from the serum in defibrinated blood or from the plasma in oxalated blood by centrifugation and were washed with a 0·9 % solution of sodium chloride. The upper layer, consisting largely of leucocytes, was removed by pipetting, suspended in isotonic saline and again centrifuged at slow speed. By repeating this process three or four times, the leucocytes were obtained free from red cells. Similarly, by pipetting the lower layer of cells and repeating the centrifugation, the red cells were obtained practically free from leucocytes.

For the enzyme tests, the red cells were laked by adding an equal volume of distilled water and allowed to remain at room temperature for 8 to 10 hours, so that the major portion of the blood-phosphoric esters became hydrolysed. The white cells were lysed with a volume of water equal to the original volume of the blood. One cc. of the lysed cells or of the cell-free serum or plasma was added to 1 cc. of a 0·2 *M* solution of sodium glycerophosphate together with a small drop of chloroform and, after adjustment of the p_H, kept at 37° for 1 hour or longer, the increase in inorganic phosphate being estimated by the Briggs method. The phosphatase content of laked red cells was not appreciably diminished by keeping the solution for periods of 6–10 days at 0° in presence of chloroform. A loss of activity amounting to about 20 % was observed in

one case after 3 weeks at 0°. Blood of rabbits, guinea-pigs and horses was used in these experiments. The red cells of horse blood had a lower phosphatase activity than those of the other animals, but this may possibly have been due to age or condition of the animal.

Attempts to purify the phosphatase of the red cells.

The separation of the phosphatase from the haemoglobin of the laked corpuscles was attempted (*a*) by precipitating the haemoglobin, leaving the enzyme in solution, (*b*) by adsorbing the enzyme on various substances. Precipitation of the haemoglobin with ammonium sulphate yielded a colourless solution, which was, however, completely inactive, both before and after dialysis. Removal of haemoglobin by precipitation as cathaemoglobin with chloroform was more successful. The laked cells were shaken with one-tenth to one-fifth their volume of chloroform and left for some hours at room temperature or 2–3 days at 0°. A considerable proportion of the pigment was thus precipitated, and by repeating this operation three or four times with fresh quantities of chloroform it was possible to remove about 95 % of the haemoglobin with a loss of only 20–30 % of the phosphatase activity. The further removal of the pigment by this method resulted, however, in rapid inactivation of the solution.

Adsorption of the phosphatase was attempted with aluminium hydroxide, kaolin, kieselguhr, barium carbonate and barium sulphate. The first three adsorbents proved satisfactory in so far that they removed a large proportion of the enzyme from the haemolysed cells, but its subsequent elution proved neither easy nor quantitative. This elution was attempted, using water, sodium phosphate solution ($M/15$) and sodium glycerophosphate solution ($M/10$) at various p_H. The best results were obtained with glycerophosphate, but even with this solution less than 20 % of the adsorbed phosphatase was recovered.

These results were not considered sufficiently good to allow the enzyme to be studied in purified condition and the experiments described below were, therefore, carried out using the crude solution obtained by laking the cells.

The optimum p_H of the blood-phosphatases.

The optimum p_H of these enzymes was first determined for the hydrolysis of glycerophosphoric ester. The p_H was measured colorimetrically by means of a capillator, a minute drop of the solution being diluted with 10 times its volume of boiled, distilled water in order to reduce the protein error. In judging the p_H of the solutions containing haemoglobin a compensating tube containing the same proportion of pigment but without indicator was used in conjunction with the standards. The results of the first experiments appeared to indicate two definite optima for the phosphatase of the red cells, one near p_H 6 and the second, less pronounced, near p_H 9. The latter was, however, found to be due to the presence of leucocytes mixed with the red cells and was no longer apparent when the cells had been more completely separated.

Table I shows the amount of sodium glycerophosphate hydrolysed by the phosphatases of red and white cells and of serum, at different p_H. In these tests the syrupy 50 % sodium glycerophosphate, which consists chiefly of the α-isomeride, was used.

The figures are calculated for 100 cc. of the lysed cells or of serum.

Table I. *Hydrolysis of glycerophosphoric ester at 37°.*

Red cells		White cells		Serum	
p_H	Hydrolysis in 5 hours mg. P	p_H	Hydrolysis in 8 hours mg. P	p_H	Hydrolysis in 10 hours mg. P
4·0	20·0	4·5	0	4·5	1·2
4·6	25·9	5·3	0	5·5	2·3
5·3	32·2	6·0	1·3	6·0	5·3
5·8	36·7	6·5	6·0	6·5	7·2
6·2	**39·0**	7·3	6·7	6·9	20·3
6·6	34·8	7·9	30·4	7·5	27·2
6·8	23·2	**8·6**	**52·0**	8·3	38·5
7·4	10·5	8·9	48·3	**8·8**	**55·9**
8·3	4·9	9·3	5·1	**9·1**	**55·8**
9·1	0·7	9·6	0·7	9·8	20·0
10·0	0·8	10·2	0	—	—

These results show that the optimum p_H for the phosphatase of the red cells is near 6·2, in agreement with the findings of Martland, Hansman and Robison [1924]; for the phosphatase of serum the optimum p_H is 8·8–9·2, which is the same as the value found by Martland and Robison [1926] and by Kay [1929, 1930, 1], but much higher than that given by Demuth [1925]. The phosphatase of the white cells has an optimum p_H 8·6–8·8, very close to that of the serum. The similarity between the optimum p_H for the phosphatases of serum and leucocytes with that of the bone phosphatase [Robison and Soames, 1924; Martland and Robison, 1927] suggests that these enzymes are probably identical. It is indeed possible that the serum phosphatase may be derived from the bones but, on the other hand, it may partly arise from the leucocytes, some of which are always broken down in blood *in vitro*.

The hydrolytic activity of the blood-phosphatases was next investigated on other substrates, namely, β-glycerophosphate, glucosemonophosphate[1], fructosemonophosphate[2], fructosediphosphate, monophenylphosphate, adenylate and guanylate[3], diphosphoglycerate[4], and the mixed acid-soluble phosphoric esters of the red blood corpuscles. The method of experiment was the same as described above, except that the amounts of the various esters added to 1 cc. of the enzyme solution were equivalent to 1·2–1·7 mg. P, instead of 6 mg. as in previous experiments. The p_H-activity curves for the three phosphatases on these different substrates are shown in Figs. 1–4, while the p_H optima are shown in Table II.

[1] Prepared by Dr R. Robison.

[2] Prepared from hexosediphosphate by Neuberg's method [1918].

[3] Prepared by hydrolysis of yeast nucleic acid and isolated in crystalline condition.

[4] Prepared from horse blood by Greenwald's method [1925].

Table II. p_H *optima of the blood-phosphatases on various substrates.*

Substrate	Phosphatase of: Red corpuscles	White cells	Serum
Sodium glycerophosphate	6·0–6·2	8·6–9·0	8·6–9·0
Sodium glucosemonophosphate	5·9–6·1	9·0–9·2	8·8–9·2
Sodium fructosemonophosphate	6·2–6·3	—	—
Sodium monophenylphosphate	5·8–6·0	8·4–8·6	8·4–8·6
Sodium adenylate	6·4–6·5	—	—
Sodium guanylate	6·4–6·5	—	—
Sodium diphosphoglycerate	6·6–6·8	8·8–9·2	8·8–9·2
Sodium fructosediphosphate	6·4–6·7	8·8–9·2	8·8–9·2
Blood-phosphoric esters	6·7–6·8	—	—

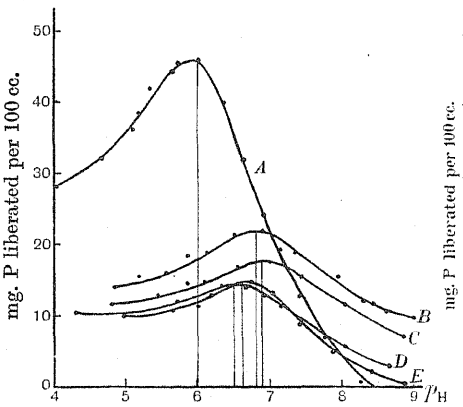


Fig. 1.

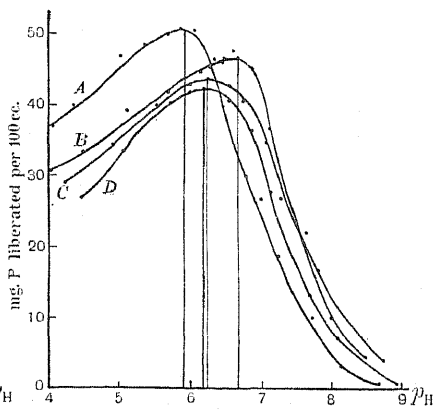
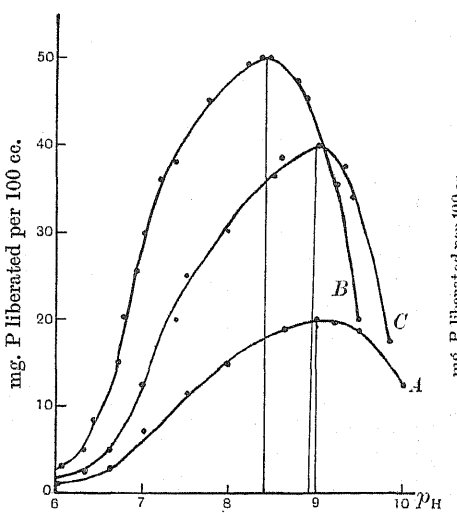


Fig. 2.

Fig. 1. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, glycerophosphate. *B*, blood-esters. *C*, diphosphoglycerate. *D*, adenylate. *E*, guanylate.

Fig. 2. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, phenylphosphate. *B*, fructosediphosphate. *C*, fructosemonophosphate. *D*, glucosemonophosphate.



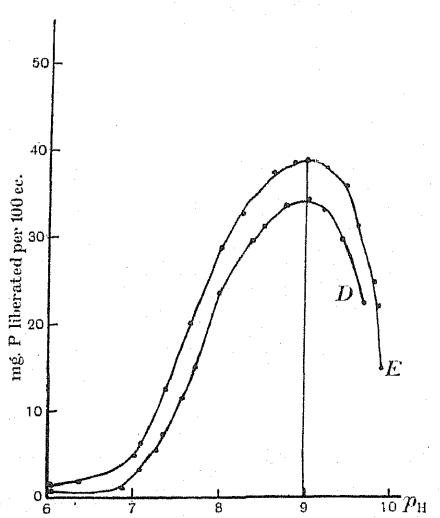


Fig. 3. p_H-activity curves for the phosphatase of the serum. Substrates: *A*, diphosphoglycerate. *B*, phenylphosphate. *C*, glycerophosphate. *D*, glucosemonophosphate. *E*, fructosediphosphate.

It is obvious from these curves that the p_H optimum for each phosphatase varies to some extent with the nature of the substrate. With the enzyme of the red cells it is lower for the monophosphoric ester (5·8–6·4) than for the diphosphoric ester (6·6–6·8). These values are close to that of p_{K_2} (6·72) for

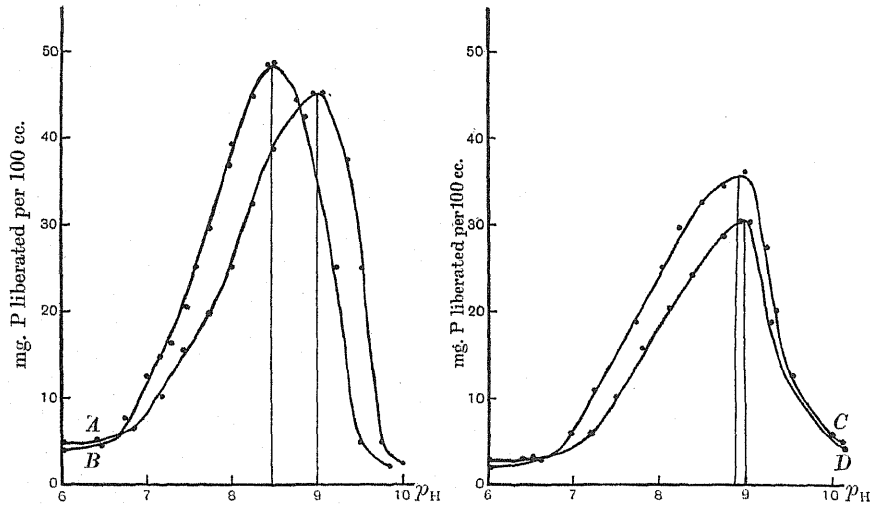


Fig. 4. p_H-activity curves for the phosphatase of the white cells. Substrates: *A*, glycerophosphate. *B*, phenylphosphate. *C*, glucosemonophosphate. *D*, fructosediphosphate.

phosphoric acid and it is possible that the observed differences are related to the differences between the dissociation constants of these esters. That the same optimum p_H was found for the hydrolysis of the blood-esters as for diphosphoglycerate is in agreement with the fact that this substance constitutes the chief part of the acid-soluble phosphorus compounds of the blood.

Such differences in the p_H optima are not so marked in the case of the serum and leucocyte phosphatases but it is possible, as was observed by Martland and Robison [1927] for the bone phosphatase, that spontaneous inactivation of the enzyme occurs at a p_H higher than 8·5, so that the apparent optimum will vary with the duration of the hydrolysis.

Specificity of the phosphatases of the blood and of other animal tissues.

The hydrolytic activity of the blood-phosphatases on various substrates was compared with that of other tissue phosphatases. The results of one typical experiment are shown in Table III. In each series of tests 1 cc. of the same enzyme preparation was allowed to act on an amount of ester equivalent to 1 mg. P at 37° and at the optimum p_H for the enzyme. The duration of the hydrolysis is shown in the Table.

The bone phosphatase was prepared according to the method of Martland and Robison [1929], and p_H 8·8 was adopted, as suggested by them, for tests with this enzyme. Intestinal and kidney phosphatases were prepared by Kay's

method [1926, 1928] and the optimum p_H 8·6, given by this author, was used in the tests.

Table III. *Hydrolysis of phosphoric esters by tissue phosphatases at their optimum* p_H *and 38° (mg. P/100 cc. of enzyme solution).*

	Source of phosphatase					
	Red cells	White cells	Serum	Bone	Intestine	Kidney
Duration of hydrolysis (hrs.)	16	10	16	1·4	1	2
Substrate (sodium salts)						
α-Glycerophosphate	38·8	32·7	27·9	47·1	38·4	15·9
β-Glycerophosphate	11·2	53·4	36·43	69·0	44·4	24·9
Glucosemonophosphate	33·7	58·2	36·9	65·2	37·9	32·8
Fructosemonophosphate	47·1	66·7	44·4	60·4	48·4	40·5
Fructosediphosphate	45·0	67·4	47·2	51·4	47·4	42·4
Monoethylphosphate	12·2	21·0	17·4	16·1	21·3	10·2
Diethylphosphate	0·1	0·1	0·1	0·2	0·2	0·1
Monophenylphosphate	75·6	68·4	63·4	75·2	48·4	39·4
Monopropylphosphate	14·4	19·7	17·1	—	—	—
Dipropylphosphate	0·1	0·2	0·1	—	—	—
Adenylate	8·2	22·1	15·1	22·2	14·0	16·9
Guanylate	9·6	20·4	16·1	25·1	15·2	14·4
Trehalosemonophosphate	5·5	22·1	16·4	20·0	6·1	18·8
Diphosphoglycerate	8·7	17·4	13·7	14·0	9·1	13·3
Pyrophosphate	6·2	31·2	7·0	39·0	35·2	15·2

Action on α- and β-glycerophosphate. Kay [1926] and Kay and Lee [1931] have shown that the kidney and plasma phosphatases attack β-glycerophosphate more readily than the α-isomeride, and the results of my experiments prove the same to be true also for the phosphatases of the leucocytes, bone and intestine and lead me to conclude that this property is common to the various tissue phosphatases for which the optimum p_H is 8·5–9·0. The phosphatase of the red cells, on the other hand, hydrolyses the α-glycerophosphate more rapidly than the β-form. It will be interesting to find whether this holds good also for the muscle enzyme which, according to Kay and Robison [1924], has an optimum p_H close to 7·0.

Action on hexosephosphoric esters. These substrates were readily hydrolysed by all the phosphatases here studied. The hydrolysis of the fructosemono- and di-phosphates was somewhat more rapid than that of glucosemonophosphate except in the case of the bone enzyme, where the relationship was reversed. It is possible that this peculiarity of the bone phosphatase may be due to the relative purity of this enzyme preparation, which contained very little protein.

Action on mono- and di-substituted phosphoric esters. Monophenylphosphate was the most rapidly hydrolysed of all the substrates that were tested. The marked effect of the substituting group on the rate of hydrolysis of the ester is shown by the difference between the results for monophenyl-, monopropyl- and monoethyl-phosphate. Another property common to all the phosphatases was their inability to hydrolyse disubstituted esters such as diethyl- and dipropyl-phosphate. The latter observation is in accordance with the previous findings of Martland and Robison [1927] for bone phosphatase, but in disagreement with those of Neuberg and Wagner [1926], who found that kidney

extract was able to hydrolyse diethylphosphate. This inability to hydrolyse compounds in which two hydroxyl groups of phosphoric acid have been esterified may, as suggested by Martland and Robison, be due to the necessity of two free hydroxyl-groups being present in the substrate in order that it may combine with the enzyme.

Action on other phosphoric esters. The purine nucleotides, trehalosemonophosphate and diphosphoglycerate were hydrolysed much more slowly than the hexosephosphates by all phosphatases.

With pyrophosphate very different rates of hydrolysis were obtained with the different enzyme preparations, which suggests that a specific pyrophosphatase may be present. It may be noted that all phosphatases were found to have some hydrolytic power on the compounds which represent the chief part of the acid-soluble phosphorus of the blood, namely, diphosphoglyceric acid [Greenwald, 1925; see also Macheboeuf, 1927], adenylic acid [Jackson, 1924; Hofmann, 1925], pyrophosphoric acid [Engelhardt, 1930; Roche, 1930] and hexosephosphoric acids [Roche, 1930]. Kay and Robison [1924] found that only a fraction, from 14 to 36 %, of these blood-esters was hydrolysed by the bone phosphatase, a result which may, perhaps, be explained by the relatively slow action of the enzyme on certain of these esters, by the inhibiting effect of inorganic phosphate or by the spontaneous inactivation of the phosphatase during the prolonged experiments.

Factors affecting the rate of hydrolysis of glycerophosphates by the phosphatases of the blood.

Martland and Robison [1927], studying the hydrolysis of glycerophosphate by bone phosphatase, observed that the velocity of this reaction was strongly diminished in presence of inorganic phosphate in concentration as low as 0·005 *M*, while the presence of the second hydrolysis product, glycerol, in concentrations up to 0·1 *M*, had no appreciable effect. Variations in the concentrations of the substrate between 0·003 *M* and 0·3 *M* likewise had no effect on the initial rate of hydrolysis. It would appear that the affinity of the enzyme for inorganic phosphate is greater than for the other constituents of the ester, and it was of interest to find whether this character is shared by the other phosphatases.

The effects of inorganic phosphate and glycerol on the rate of hydrolysis of glycerophosphate (mixed α- and β-forms) by phosphatases of the red cells and of serum are shown in Table IV.

The retarding influence of inorganic phosphate on the rate of hydrolysis is very marked with both these phosphatases, while glycerol also has a considerable inhibitory effect, especially in the case of the red cell phosphatase. The degree of retardation increases with increasing concentration of both hydrolysis products.

Table V shows the effect of variations in the concentration of the substrate on the rate of hydrolysis.

Table IV. *Effect of inorganic phosphate and glycerol on the hydrolysis of glycerophosphate by phosphatases of red cells and serum.*

Initial concentration of ester 0·05 *M*. $t = 37°$. Hydrolysis calculated as mg. P per 100 cc.

Red cells, p_H 6·3	Initial concentration of inorganic phosphate (*M*)					
Hours	*0·03*	*0·015*	*0·01*	*0·005*	*0·003*	*0·002*
1½	2·1	3·3	4·5	7·7	9·7	11·1
3	5·2	7·0	7·8	11·3	13·6	16·0
4½	8·5	10·6	11·0	14·6	18·1	20·1
8	13·3	14·6	16·1	20·8	24·7	27·9
Serum, p_H 8·6	*0·025*	*0·013*	*0·007*	*0·003*	*0·002*	
4½	5·3	6·7	7·5	8·5	8·9	
10	7·9	9·5	10·4	11·7	14·0	
Red cells, p_H 6·3	Initial concentration of glycerol (*M*)					
	0·1	*0·02*	*0·01*	*0·005*	*0*	
3	6·1	10·5	16·2	17·0	19·1	
6½	11·2	16·0	25·1	26·3	31·5	
Serum, p_H 8·6						
4	1·7	2·2	3·4	5·0	6·1	
8	4·3	4·4	8·1	9·1	11·3	

Table V. *Effect of concentration of substrate on the rate of hydrolysis of glycerophosphate.*

Red cells		Serum	
Concentration of ester (*M*)	Hydrolysis in 2 hrs. at p_H 6·3 and 37° (mg. P per 100 cc.)	Concentration of ester (*M*)	Hydrolysis in 6 hrs. at p_H 8·6 and 37° (mg. P per 100 cc.)
0·0075	1·4	0·006	5·9
0·015	2·9	0·013	6·9
0·030	9·4	0·032	7·0
0·075	23·0	0·064	10·2
0·150	37·0	0·128	11·8
—	—	0·320	14·9

With both enzymes the rate of hydrolysis was increased by raising the concentration of substrate between the limits shown in Table V, but, whereas in the case of the serum phosphatase the effect was comparatively small, with the red cell phosphatase the rate of hydrolysis was nearly proportional to the initial concentration of ester. The difference between the behaviour of the serum and bone phosphatases may be due to the specific characters of these enzymes or to the very different state of purity of the preparations used.

The synthetic action of the blood-enzymes.

The synthetic action of bone phosphatase has been demonstrated by Martland and Robison [1927] and that of kidney phosphatase by Kay [1928]. In Table VI the results of a number of experiments are set out, showing synthesis of phosphoric esters effected by the phosphatases of the red cells and of serum in presence of various alcohols. Sodium fluoride in 1 % concentration had no significant effect on such synthesis.

Table VI. *Synthesis of phosphoric esters by blood-phosphatase.*

Alcohol	Inorganic phosphate (mg. P per 100 cc.) Days 0	2	5	10	18	Synthesis (mg. P per 100 cc.)
Serum phosphatase (p_H 8·5: t. 37°)						
Glycol 45 %	159·2	137·2	116·2	115·4	115·4	43·8
Glycerol 40 %	300·2	300·0	296·9	284·9	273·4	26·8
,,	244·4	238·4	—	219·6	199·8	44·6
,,	125·4	117·2	110·2	106·4	108·4	17·0
Sorbitol (saturated)	157·6	156·3	—	—	152·2	5·4
Dulcitol (saturated)	160·1	159·4	—	—	157·4	2·7
Erythritol (saturated)	158·4	160·1	159·2	158·4	157·7	0·7
Glucose 50 %	138·4	139·2	116·9	110·2	109·4	29·0
,, 50 % + 1 % NaF	134·9	134·2	114·4	109·2	106·4	28·5
Fructose 50 %	115·1	112·4	107·6	100·4	92·4	22·7
,, 50 % + 1 % NaF	112·1	113·4	110·4	113·2	92·2	19·9
Glyceric acid 30 %	220·2	218·2	199·9	194·6	192·9	27·3
Red cell phosphatase* (p_H 6·3: t. 37°)						
Glycol 45 %	158·1	144·2	—	130·4	124·4	23·7
Glycerol 40 %	298·7	304·4	—	294·4	270·8	27·9
,,	239·2	244·4	226·4	194·4	190·6	49·6
,,	128·3	130·2	—	122·1	120·1	8·2
Sorbitol (saturated)	158·4	164·4	—	—	160·2	—
Dulcitol (saturated)	159·7	162·4	—	159·4	159·1	—
Erythritol (saturated)	155·4	160·2	—	159·8	160·0	—
Glucose 50 %	127·2	130·6	124·4	118·4	113·4	13·8
,, 50 % + 1 % NaF	121·1	124·6	120·3	114·4	110·4	10·7
Fructose 50 %	121·4	123·4	120·2	116·4	115·3	6·1
,, 50 % + 1 % NaF	127·4	128·9	126·4	120·2	120·1	7·3
Glyceric acid 30 %	195·1	203·2	196·4	—	193·4	1·7

* The slight increase in inorganic phosphates after 2 days' experiment is probably due to the hydrolysis of blood-esters.

Summary.

1. The phosphatases present in serum and in the red and white cells of the blood have been studied and some of their properties compared with those of the bone, kidney and intestinal phosphatases.

2. The phosphatase of the red cells is quite distinct from all the other enzymes. It differs from them in having an optimum p_H on the acid side of neutrality, 6·0–6·8, according to the substrate, and in acting more energetically on α- than on β-glycerophosphate.

3. The phosphatases of the serum and of the white cells appear to be identical, while the small differences observed between the properties of these enzymes and of the bone phosphatase may probably be attributed to differences in the state of purity of the preparations examined. Kidney and intestinal phosphatases are also probably identical with these.

4. The relative rates of hydrolysis of various substrates by these phosphatases have been measured. All monosubstituted phosphoric esters examined were acted on by each enzyme, but the rate of hydrolysis varied with the nature of the substituting group. Disubstituted esters were not appreciably hydrolysed by any of these phosphatases.

5. The hydrolysis of glycerophosphate by each of the blood-phosphatases is diminished by the presence of inorganic phosphate and also by glycerol.

6. The phosphatases of the serum and red cells are able to synthesise phosphoric esters from inorganic phosphate and various alcohols, including glycol, glycerol, and hexoses.

I wish to express my thanks to the Director of the Lister Institute and to Prof. A. Harden for the hospitality of the Biochemical Department and to Dr R. Robison for his interest and helpful criticism in this work. I wish also to thank the Rockefeller Foundation for the Fellowship which enabled me to carry out this research.

REFERENCES.

Demuth (1925). *Biochem. Z.* **159**, 415.
Engelhardt (1930). *Biochem. Z.* **227**, 16.
Greenwald (1925). *J. Biol. Chem.* **63**, 339.
Hofmann (1925). *J. Biol. Chem.* **63**, 675.
Jackson (1924). *J. Biol. Chem.* **59**, 529.
Kay (1926). *Biochem. J.* **20**, 791.
—— (1928). *Biochem. J.* **22**, 855.
—— (1929). *Brit. J. Exp. Path.* **10**, 253.
—— (1930, 1). *J. Biol. Chem.* **89**, 235.
—— (1930, 2). *J. Biol. Chem.* **89**, 249.
—— and Lee (1931). *J. Biol. Chem.* **91**, 135.
—— and Robison (1924). *Biochem. J.* **18**, 1139.
Macheboeuf (1927). Thèse Med. Paris.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
—— and Robison (1926). *Biochem. J.* **20**, 847.
—— —— (1927). *Biochem. J.* **21**, 665.
—— —— (1929). *Biochem. J.* **23**, 237.
Neuberg (1918). *Biochem. Z.* **88**, 432.
—— and Wagner (1926). *Biochem. Z.* **171**, 485.
Robison (1923). *Biochem. J.* **17**, 286.
—— and Soames (1924). *Biochem. J.* **18**, 740.
Roche (1930). *Bull. Soc. Chim. Biol.* **12**, 636.
—— and Roche (1929). *Bull. Soc. Chim. Biol.* **11**, 549.

CLXXXVII. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. II.

By STANISLAUS JOHN PRZYŁĘCKI
and WENCESLAUS GIEDROYĆ.

From the Biochemical Laboratory, Faculty of Medicine, The University, Warsaw.

(*Received May 29th, 1931.*)

Fresh evidence will be given in this paper in support of the opinion that the free charge of protein particles in solution is dependent on the presence of neutral salts and on their concentration; in particular on the valency of their anti- and iso-ions.

It was shown in our previous communication [1931] that, as demonstrated by experiments on flocculation induced by heat or by addition of alcohol, proteins may be discharged at p_H other than that of the isoelectric point by the addition of a critical concentration of electrolyte, termed in our previous paper C_K. Experiments will be described in this paper, showing that at concentrations greater than the critical the protein particles may, at the same p_H, be supercharged, so that protein solutions are obtained in which, at p_H values smaller than that of the isoelectric point, the particles behave as anions, whilst at p_H higher than the isoelectric they behave as cations. The possibility of such supercharging was indicated by Kruyt's [1929] experiments on the action of sodium sulphate on the cataphoretic properties of gelatin at p_H 3·9, as well as by the work of Loeb [1924, 1925].

Experimental.

1. Gelatin.

Gelatin was purified by the method described in the preceding paper, and the flocculation of aqueous solutions of such gelatin, in the presence of 60–70 % of alcohol, was studied as before. Since it appeared probable that supercharging could most readily be achieved by the addition of electrolytes to solutions at a p_H differing little from that of the isoelectric point, hydrochloric acid was added to p_H 3·5–3·8, or sodium hydroxide to p_H 6·5–7·0.

1 % gelatin solutions at p_H 3·5 were treated with an excess (0·001–0·004 M) of potassium ferricyanide, the C_K of which is about 0·0001 M at this p_H. In the presence of alcohol (70 %) gelatin is quantitatively precipitated in the presence of 0·001 M $K_3Fe(CN)_6$, whilst when excess of this reagent is taken, 10–20 % of the gelatin remains in solution. Filtrates from such systems were taken for examination.

Various salts (sodium sulphate, potassium ferricyanide, calcium and magnesium chlorides) were added to these filtrates. The results obtained indicate that gelatin treated with an excess of ferricyanide at p_H 3·5 becomes practically insensitive to the action of multivalent anions, but is readily flocculated by salts giving rise to univalent anions and bivalent cations. Thus ordinarily the C_K of sodium sulphate is less than 0·001 M, and of magnesium chloride greater than 0·05 M, whilst supercharged gelatin is not flocculated by the addition of 0·02 M sodium sulphate, and is coagulated by 0·0005 M magnesium chloride.

Similar experiments were performed on alkaline gelatin solutions, at p_H 6·5–7·0, adding excess of neutralised aluminium chloride (0·002–0·005 M), when a precipitate formed, the bulk of which was increased by the addition of alcohol to 70 %. The filtrate contained gelatin which was sensitive to the action of multivalent anions, but not cations. Further addition of aluminium chloride did not lead to flocculation of the residual gelatin, and the same applies to magnesium chloride, the C_K of which is normally 0·0025 M, whilst in this case flocculation is not obtained using 0·02 M solutions. On the other hand, the C_K of sodium sulphate is now 0·0003–0·0008 M as compared with the normal value of over 0·01 M.

2. *Serum-albumin.*

A similar sensitisation to the action of cations and desensitisation to that of anions is obtained using serum-albumin solution at p_H 3·5 by the addition of excess ferricyanide (0·001–0·005 M), and, conversely, sensitisation to the action of anions and desensitisation to that of cations can be demonstrated in neutral solutions (p_H 6·5–7·0) by the addition of 0·01–0·02 M aluminium chloride. The results obtained are based on observations of coagulation after heating 10 min. at 100°.

REFERENCES.

Kruyt (1929). *Kolloidchem. Beih.* **29**, 347.
Loeb (1924). Les protéines. (Alcan, Paris.)
—— (1925). La théorie des phénomènes colloidaux. (Alcan, Paris.)
Przyłęcki (1931). *Biochem. J.* **25**, 713.

CLXXXVIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

IX. THE SYNTHESIS OF PHENYL SULPHATE AND INDOXYL SULPHATE.

By THOMAS SHIRLEY HELE.

From the Biochemical Department, University of Cambridge.

(*Received September 1st, 1931.*)

The theory that the organic or ethereal sulphates arise from phenols by conjugation with sulphur compounds other than sulphate, oxidation occurring subsequently, rests entirely on negative evidence. In more recent years Rhode and Sherwin have supported this theory and Sherwin has gone further, suggesting that ethereal sulphates are formed only from sulphur-containing complexes of endogenous origin. Rhode [1922] in experiments on rabbits, and Sherwin in collaboration with Shiple and Muldoon [1924] in experiments on a pig failed to show any union between phenols and sodium sulphate administered concurrently. They made use of phenol itself and of certain halogenated phenols. The chief evidence for the older theory [Baumann, 1876], that ethereal sulphates arise by the combination of phenols with preformed sulphate, lies in the ability of the dog to bring about the union of guaiacol and the sulphate ion of sodium sulphate [Hele, 1924, 1]. In discussing these differences Pryde [1931] suggests that "guaiacol is a substance too far removed from the normal metabolic paths to warrant comparison with phenol in this respect." He makes no comment however on differences in animal species or differences in experimental technique.

The pig, unlike the dog, does not utilise sulphur compounds readily in detoxication processes and this may also be true of the rabbit [Coombs and Hele, 1926; Lawrie, 1931]. Under such circumstances to demonstrate the truth of Baumann's theory becomes a matter of extreme difficulty. No one has succeeded in proving it in either rabbit or pig, but failure to do so does not mean proof to the contrary. Apart however from the question of species Rhode and Sherwin with his co-workers, employing phenols of considerable toxicity and in relatively small doses, did not produce experimental conditions that could be successful. In my experiments the dog was used as the most suitable animal and guaiacol was chosen on account of its low toxicity, when given orally in the form of carbonate. The dog, as it were, could be saturated with guaiacol without any effect on its health. All sulphate arising from the tissues or the diet was used up in the detoxication process. The demand for

sulphate was greater than the supply, so that sodium sulphate, added to the diet, was immediately utilised by the animal.

In the present paper evidence is brought forward to show not only that the dog is capable of utilising sodium sulphate in the synthesis of phenyl sulphate and of indoxyl sulphate but also that, when the same syntheses are brought about at the expense of endogenous sulphur, the sulphur so utilised is in the form of the sulphate ion [Hele, 1927].

Experimental.

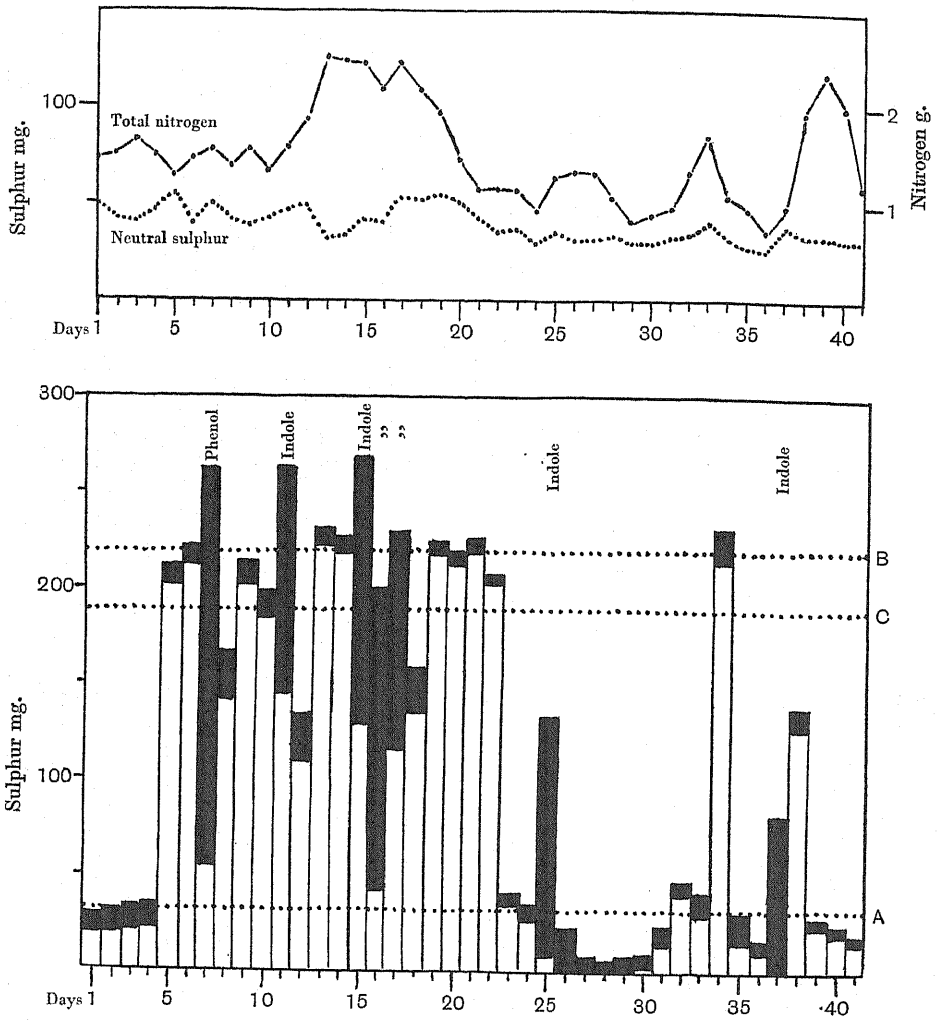
The experimental methods employed in this research have already been described [Hele, 1924, 2]. Two bitches were used, Patsy (6·8 kg.) and Pansy (9·0 kg.). As repeated doses of phenol could not be given safely, the "saturation" method, successfully utilised in the case of guaiacol, had to be discarded. The animals therefore were dosed daily with sodium sulphate and on certain days phenol or indole was administered orally in amounts sufficient to show an effect on the sulphur metabolism. This method of dosage had already successfully demonstrated the capacity of the dog to use sodium sulphate in the formation of chlorophenyl sulphate from chlorobenzene [Hele, 1924, 2]. In order to make the animal dependent for the formation of ethereal sulphate either on its endogenous sulphur or on the sodium sulphate, the protein of the diet and therefore the sulphate, arising therefrom, was reduced to the minimum. The quantity of meat retained in the diet was only just sufficient to place round the gelatin capsules, containing the phenol or indole on dose days. The meat was kept in position round the capsules by a few cotton stitches. Calories were provided in the form of margarine and sucrose. The actual amounts were determined experimentally by the quantities the bitches were prepared to swallow over long periods of time. The number of calories was inadequate and the bitches lost weight. It was found desirable to retain some milk in the diets.

Pansy's diet was as follows:

		Total N g.	Total S g.	Calories K
Meat:	10 g.	0·34	0·023	13
Sugar:	60 g.	—	—	246
Margarine:	50 g.	—	—	400
Milk:	50 cc.	0·26	0·023	36
Water:	100 cc.	—	—	—
	Total	0·60	0·046	695

Patsy received only 40 g. of margarine but in other respects her diet was identical with that of Pansy.

When administered, the daily dose of sodium sulphate was 2 g. of the decahydrate (containing 200 mg. of sulphur), half given at 9.30 a.m., half at 5 p.m., either in the milk of the diet or subcutaneously, dissolved in 5 cc. of water, and the daily dose of phenol or indole was 1/100 of the g. molecular



Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 1. "Patsy."

Days	5–22	2 g. sodium sulphate, cryst. orally, daily.
Day	34	2 g. " " " "
"	38	2 g. " " " "
"	7	1 × M.W./100 g. phenol, orally.
"	11	1 × M.W./100 g. indole, "
Days	15–17	1 × M.W./100 g. " " daily.
Day	25	1 × M.W./100 g. " "
"	37	1 × M.W./100 g. " "

Line A.	Daily sulphate sulphur on diet	32 mg.
" B.	" " " on diet and sodium sulphate	220 mg.
" C.	B less A	188 mg.

Patsy's weight fell from 6·8 to 6·0 kg. in 41 days.

weight (sufficient to combine with 320 mg. sulphur as sulphate), half given orally at 9.30 a.m., half at 5 p.m. The urine was collected from 9 a.m. to 9 a.m.

The first experiment carried out under these conditions was immediately successful. It is shown diagrammatically in Fig. 1. The excretion of total nitrogen and of neutral sulphur is shown in simple curve form. The excretion of sulphate is shown in column form in order to exhibit certain quantitative relations. The black portion of each column represents ethereal sulphate and the white portion inorganic sulphate. The average output of sulphate-sulphur on diet only was 32 mg. Line *A* represents this value. The average output of sulphate-sulphur on sodium sulphate days was 220 mg. (line *B*). On day 7 phenol was given orally and it is manifest that the major portion of the excreted sulphur was in the form of ethereal sulphate. In the formation of ethereal sulphate, it is best to assume that the sulphur first utilised is derived from all sources other than the administered sulphate. Line *C* has therefore been drawn at level 188 mg. S (220 mg. less 32 mg.). The black portion of the column is thus divided into three parts:

(1) a part above line *B* (43 mg. S), in excess of daily sulphate output and therefore derived from the tissues;

(2) a part between lines *B* and *C* (32 mg.), representing the daily output of sulphate derived from the diet and the tissues;

(3) a part below line *C* (133 mg. S), derived from the sodium sulphate.

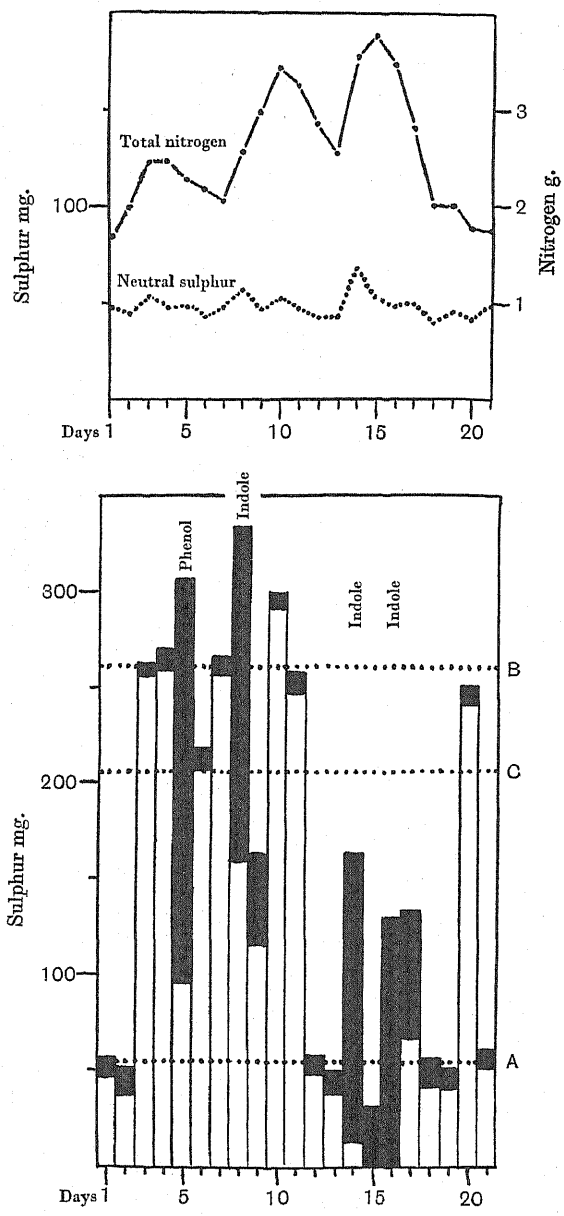
At least 66 % of the administered sodium sulphate has been utilised.

On day 8 the output of sulphate is less. 53 mg. S from some source or other, normally excreted as sulphate, has been retained. The amount retained corresponds in some measure to the excess excreted on the dose day. Presumably the one is the consequence of the other.

A result, similar though not so good, follows on the administration of indole on the eleventh day.

By repeating the daily doses of indole over a period of three days (days 15–17) it was hoped that a greater drain on the endogenous sulphur would result. However there was actually some retention of sulphur on the second day of the series and the net excess sulphur excreted over the 3 days was only 39 mg., a figure very like those obtained for single day doses. It therefore appears that the amount of endogenous sulphur, immediately available for the formation of ethereal sulphate, is limited in amount. The retention of sulphur, following the dose days, was 61 mg.

It was next decided to attempt the replacement of the sulphur lost on dose days by the sulphate of the sodium sulphate. On day 25 indole was administered without sodium sulphate. There was an increased sulphate-sulphur excretion of 102 mg. To make good this loss sulphur was retained for the following 5 days, the total being 103 mg. This must have been derived partly from the food and partly perhaps from the tissues. On day 7 indole was again given alone and on the following day sodium sulphate. On the indole day 50 mg. excess sulphate-sulphur was excreted and on the sulphate



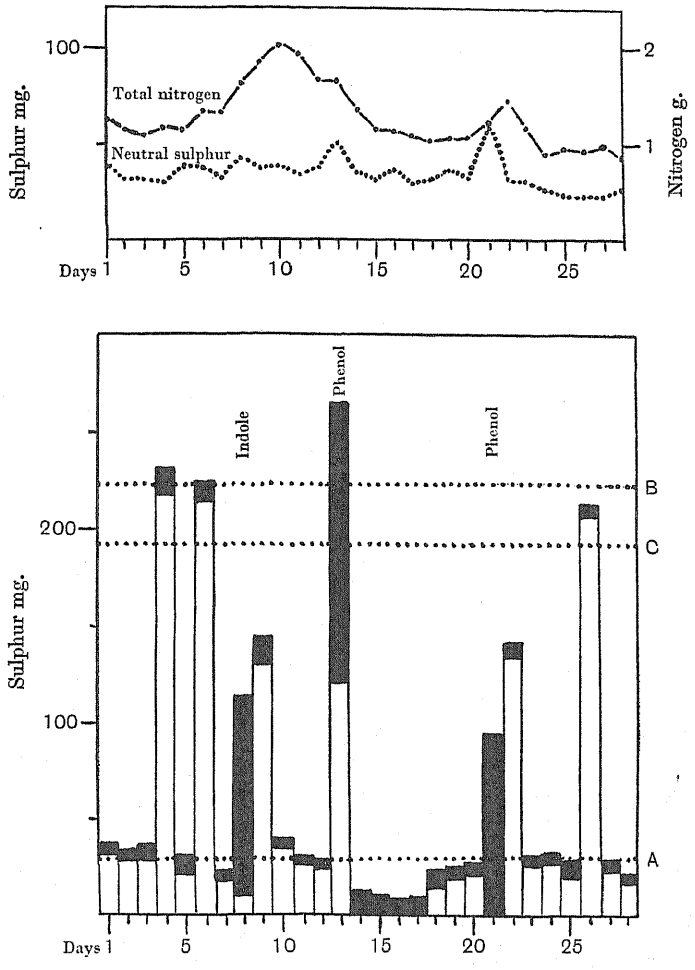
Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 2. "Pansy."

Days 3–11 2 g. sodium sulphate, cryst. orally, daily.
Day 17 2 g. ,, ,, ,, ,,
,, 20 2 g. ,, ,, ,, ,,
,, 5 1 × M.W./100 g. phenol, orally.
,, 8 1 × M.W./100 g. indole, ,,
,, 14 1 × M.W./100 g. ,, ,,
,, 16 1 × M.W./100 g. ,, ,,

Line A. Daily sulphate-sulphur on diet ... ... 55 mg.
,, B. ,, ,, ,, on diet and sodium sulphate 262 mg.
,, C. B less A ... ... 207 mg.

Pansy's weight fell from 9·0 to 8·3 kg. in 21 days.



Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 3. "Patsy."

Day	4	2 g. sodium sulphate, cryst. subcutaneously.
,,	6	2 g. ,, ,, ,, ,,
,,	9	2 g. ,, ,, ,, ,,
,,	13	2 g. ,, ,, ,, ,,
,,	22	2 g. ,, ,, ,, ,,
,,	26	2 g. ,, ,, ,, ,,
,,	8	1 × M.W./100 g. indole orally.
,,	23	1 × M.W./100 g. phenol ,,
,,	21	1 × M.W./100 g. ,, ,,

Line A.	Daily sulphate-sulphur on diet	30 mg.
,, B.	,, ,, ,, on diet and sodium sulphate	223 mg.
,, C.	B less A	193 mg.

Patsy's weight fell from 6·8 to 6·5 kg. in 28 days.

day 81 mg. sulphur were retained. Immediately afterwards the sulphate excretion returned to normal. Even if it is assumed that 32 mg. of the sulphur retained were derived from the normal daily output of sulphate, a balance of 49 mg. is left, which must have come from the administered sodium sulphate.

Similar experiments were carried out on "Pansy." Fig. 2 has been constructed on the same lines as Fig. 1. The results obtained by the administration of phenol (day 5) and of indole (day 8) are similar to those obtained in Exp. 1. In the latter case however there are certain discrepancies. The apparent retention of sulphur on day 9 is extremely great and on day 10 the excretion of sulphate is much in excess of the normal. The most likely explanation of these discrepancies is the delayed absorption and therefore the delayed excretion of the sulphate given on day 9. If the excess sulphate on day 10 is added to the sulphate on day 9, the figure for the retained sulphur on that day becomes 62 instead of 100 (see Table I). On days 14 and 16 indole was administered without sodium sulphate, again with the intention of draining the animal of all sulphur available for the synthesis of ethereal sulphate. The excess excretion on the two days was 184 mg. S. Sodium sulphate was administered on day 17. The sulphur retained on days 15 and 17 was 151 mg., of which at least 73 mg. must have been derived from the sodium sulphate.

In Exp. 3 (Fig. 3) the sodium sulphate was given subcutaneously, to eliminate the possibility of any of the sulphate failing to reach the tissues. Indole was given on day 8 and the excess sulphate-sulphur, excreted on this day, was 84 mg. Sodium sulphate was administered on the following day and the sulphur retained on that day was 78 mg. The corresponding figures for phenol (days 21, 22) were 65 and 81. On day 13 phenol was given with sodium sulphate and the excess sulphate-sulphur excreted on that day was 42 mg. The sulphur retained on the four following days was 77 mg. 37 % of the administered sodium sulphate was utilised in the formation of the phenol sulphate. It makes no difference therefore whether the sodium sulphate is given orally or subcutaneously.

The calculations are based on averages for normal diet and normal sulphate days and are therefore subject to error[1]. The nitrogen output in each experiment and to a lesser degree the neutral sulphur output varied most markedly, but in most instances these variations do not seem related to variations in the sulphate output. However, the corresponding variations in the sulphate excretion, if they occur, would be small and would be masked by the much greater variations due to the administration of the sodium sulphate, of the phenol and of the indole. On the whole it is somewhat remarkable that the calculations bear out the main thesis of this paper so consistently as they do.

[1] For example: in Exp. 2 line C is absolutely too high; it is drawn at the level of 207 mg. S and actually only 200 mg. S were given as sodium sulphate on sulphate days.

Discussion.

In calculating the figures, which appear in Table I, two assumptions have been made—that the administered sodium sulphate is the last source of sulphur to be used (1) in the formation of ethereal sulphate and (2) in making good the sulphur lost from the tissues on dose days. The figures given therefore in the Table under sodium sulphate are minimum values.

Table I.

Day	Substance	Percentage phenol or indole excreted on dose day as ethereal sulphate	Ethereal sulphate excreted on dose day: From excess sulphur mg. S	From sulphur of diet mg. S	From sodium sulphate mg. S	Total mg. S	Percentage sodium sulphate utilised	Sulphur retained after dose day mg. S	Minimum amount of sodium sulphate retained mg. S
Exp. 1:									
7	Phenol	62*	43	32	133	208	66	53	21
11	Indole	34*	44	32	43	119	22	85	53
15–17	Indole	40*	39	96	278	413	46	61	29
25	Indole	36*	102	24	—	126	—	103	—
37	Indole	23	50	32	—	82	—	81	49
Exp. 2:									
5	Phenol	63	45	55	112	212	56	43	—
8	Indole	50*	71	55	49	175	25	100	45
14	Indole	41*	109	34	—	143	—	23	—
16	Indole	37*	75	55	—	130	—	128	73
Exp. 3:									
8	Indole	30	84	20	—	104	—	78	48
13	Phenol	43	42	30	73	145	37	77	—
21	Phenol	27	65	30	—	95	—	81	51

* Some in addition was also excreted on the day following the dose day.

It is clear from the Table that 23 to 62 % of the phenol or indole administered has been excreted on the dose day in the form of ethereal sulphate and at least 22 to 66 % of the sodium sulphate given has been utilised in the synthesis of the ethereal sulphate. One cannot expect all the administered sulphate to be utilised. Some will always escape through the kidney, before the synthesis can take place. Also not all the phenol or indole is available for the sulphate synthesis; part is excreted with glycuronic acid and part is probably oxidised. The utilisation of the sodium sulphate has been as full as can be expected. The mode of formation of ethereal sulphate from phenol or indole is the same as that from guaiacol.

In the Table are also shown the supposed sources of origin of sulphur, taking part in the ethereal sulphate synthesis. In column 4 are given the amounts of excess sulphur excreted on dose days. Such sulphur would commonly be called endogenous. Some persons might demur to attaching such a label to small amounts of excess sulphur, amounts that might be regarded as exogenous products delayed in excretion. When however the excess figures become large, it would be difficult to avoid the term endogenous. In the Table the corresponding figures for retained sulphur are also given and in the last column from these have been deducted the normal sulphate output

on diet days. The balance must represent the minimum amounts of sodium sulphate retained to make good the loss of sulphur from the tissues.

The most likely explanation of these experimental facts is the simplest. There is no reason to suppose that there are any differences in this particular respect in the oxidation products of endogenous and exogenous sulphur. It is unnecessary to distinguish between the two lines of metabolism. It is enough to say that sulphate arises by oxidation of sulphur compounds in organs. Before the sulphate is finally excreted by the kidneys, it accumulates in the body fluids. In a small dog this accumulation amounts to about 50 to 100 mg. S.

In the detoxication of phenols the first sulphur to be used is probably the sulphate ion in the cell fluids. As the immediate supply of sulphate is used up, it is replaced by oxidation products of metabolism. When the body fluids are depleted of sulphate by the administration of phenols, sulphate accumulates slowly over a series of days as it arises in the course of metabolism or as it can be replaced by sulphate in the diet, until the concentration is such that excretion again becomes normal. It seems likely that the sulphate excreted in excess on dose days and the sulphate retained afterwards are present in the tissues in the ionised condition and not in combination and undissociated, but of this there is no proof. To some the retention of sulphate by the animal may seem remarkable but it must be remembered that the concentrations involved are no greater than the concentration of sulphate (2 to 3·5 mg. S per 100 cc.) in normal dogs' blood [Denis, 1921]. If the sulphate retained were distributed uniformly (a more than improbable assumption) throughout the body fluids, the concentration would be about 1 to 2 mg. S per 100 cc.

Summary.

1. The dog can utilise sodium sulphate, given orally or subcutaneously, to form phenyl sulphate and indoxyl sulphate from phenol and indole respectively.

2. When ethereal sulphate is formed by the dog from phenol or indole at the expense of endogenous sulphur, this sulphur can be replaced at least in part by the sulphate ion.

3. It is suggested that in every case ethereal sulphate, whether arising endogenously or exogenously, is formed by the union of the sulphate ion with phenolic compounds.

The expenses of this research were largely defrayed out of grants from the Royal Society.

REFERENCES.

Baumann (1876). *Arch. ges. Physiol.* **13**, 285.
Coombs and Hele (1926). *Biochem. J.* **20**, 606.
Denis (1921). *J. Biol. Chem.* **49**, 315.
Hele (1924, 1). *Biochem. J.* **18**, 110.
—— (1924, 2). *Biochem. J.* **18**, 586.
—— (1927). *Chem. Ind.* **46**, 937.
Lawrie (1931). *Biochem. J.* **25**, 1037.
Pryde (1931). Recent advances in biochemistry, 3rd Edition, p. 49. (Churchill, London.)
Rhode (1922). *Z. physiol. Chem.* **124**, 15.
Shiple, Muldoon and Sherwin (1924). *J. Biol. Chem.* **60**, 59.

CLXXXIX. INVESTIGATIONS ON THE DETERMINATION OF VITAMIN D.

COMPARISON BETWEEN THE PREVENTIVE AND THE CURATIVE METHODS.

By POUL SCHULTZER.

From the University Institute of Hygiene and the Budde Laboratory, Copenhagen.

(*Received July 8th, 1931.*)

Introduction.

For the biological standardisation of vitamin D, several fundamentally different methods have been devised, but only three of these are in use. These methods are based on the presence or absence of rickets in young rats kept on a rachitogenic diet *plus* a varying addition of vitamin D. The preventive method consists in giving the vitamin D addition to the experimental animals at the same time as they are placed on the rachitogenic diet. The curative method implies the supposition that after rickets is established in the animals they are cured of this lesion by a suitable addition of vitamin D to the rachitogenic diet. Not all investigators insist upon complete recovery, however, but merely a certain degree of recovery. In these two methods the decisive point is, whether at the end of the experiment the animals have rickets or not; yet, as mentioned, a slight degree of rickets may be allowed in tests by the curative method. To decide whether rickets is present or to what extent the condition of rickets has improved, the various authors have used different methods. Macroscopic pathological-anatomical examination is the simplest method, but most authors are agreed that this is relatively rough. It may be supplemented by histological examination of the bones, but this complicates the test considerably.

X-ray examination is just as simple as the macroscopic pathological-anatomical examination. It was first employed by American investigators and it has the great advantage that it is practicable on the living animal. Systematic X-ray examination has been elaborated by Poulsson and Løvenskiold [1928], and others. Methods based on chemical analysis are somewhat more circumstantial, whether concerning the dry substance, ash (including Ca and P) of the bones or the calcium and phosphorus content of the serum.

Of these methods of examination it may be said that the investigators working with them have obtained results they consider satisfactory.

Weighing the two methods, it seems obvious to me that the preventive

method offers several advantages over the curative. It takes less time for the experiment. Moreover a "preventive dose" is more precise than a "curative dose," as several authors differ in their requirements as to the time in which recovery, or relative recovery, is to take place.

The third method to be mentioned in this paper is Shipley's line test [McCollum, Simmonds, Shipley and Park, 1922]. In the last two years considerable attention has been paid to this method. In this method it is not the recovery of rachitic young rats that decides the estimate; the essential feature of the line test is a sign of commencing recovery, namely, the precipitation of calcium phosphate, demonstrable with silver nitrate under influence of the light as a blackening around the epiphyseal lines (tibia). The method has been the subject of thorough investigations [Steenbock and Black, 1925; Coward, 1928; Adams and McCollum, 1928; Bills *et al.* 1931], and judging from these, it appears to be serviceable. Still, several objections may be raised to this method—objections which have indeed been foreseen by several of the authors mentioned, without, I think, being sufficiently refuted.

One weighty objection is that the line test may give a positive result in conditions other than commencing recovery. As has been demonstrated first by Cavins [1924], in young rats there appears on inanition a very distinct precipitation of calcium salts at the epiphyseal line. This precipitation is so marked that it can be demonstrated with X-rays. Of course, the vitamin tests in question do not involve any true inanition, but from the papers quoted it is evident that the weight curve of rachitic young rats will often show a marked change when the diet is supplemented with an addition, for instance, of cod-liver oil. Thus, Adams and McCollum give several instances of such an influence upon the weight curve, the animals showing a natural rise of weight during the fore-period and a loss of weight during the test period. Quite naturally, one will think that these instances involve at least a partial inanition. In going through the tables of Adams and McCollum, it is found in keeping with this, that the most positive results of the line test are obtained in the rats that are losing weight. Of course, rats that are losing weight may be left out of consideration, as well as the rats with a strikingly small weight during the test period; and, in fact, Bills *et al.* [1931] recommend such a procedure. But a method that allows of exclusion of test animals according to arbitrary criteria involves a certain degree of unreliability, and should, therefore, be avoided as far as possible.

Nor is there any doubt that a quantitative estimation of the line test is difficult. I am here leaving out the macroscopic estimation and refer to the microscopic examinations reported by Adams and McCollum and by Bills *et al.* They show that in the line test the histological picture is rather polymorphous. The calcium precipitation may take place in several ways. The last scheme worked out by Bills must be said to show a distinct gradation of the precipitation phenomena. But the fact that there is a rather wide scattering in the results of the line test in animals getting the same addition

makes it evident that the significance of this gradation may still be rather problematic; and it is also questionable whether Bills is justified in working with the average of his arbitrary gradation. Besides, as mentioned before, some animals have already been left out on account of their weight.

Experimental.

In the following I have tabulated a number of experiments by the preventive and the curative methods; and I have also tried to include the line test in these comparative examinations. In the preventive method that dose of cod-liver oil is designated as preventive which is the smallest of the doses employed that is sufficient to keep the test animals free from rickets. In the curative method, the curative dose of cod-liver oil is the dose which is just enough to give the greatest effect, while greater doses have no additional effect under the given experimental conditions.

Technique.

The experiments were made on young white rats, 30 days old, taken direct from the mother. The stock of breeding animals has been kept through 10–11 generations on Gudjónsson's diet No. 4 [1930], so there have been but comparatively slight variations in the size of the litters and the average weight of the young.

Gudjónsson's diet No. 4 consists of:

Skimmed-milk powder	30 %
Rice flour	40 %
Autolysed yeast	15 %
Coconut oil (with 0·3 % shark-liver oil) ...	15 %

The young rats are transferred from the stock quarters to the experimental room, where daylight is always shut out. The temperature of this room has been recorded by means of a thermograph, and throughout all the experiments it has been 20–25°. Each animal has been alone in its cage. In all cases the diet in the experimental period has been McCollum's diet No. 3143, which consists of:

Whole wheat	33 parts
Whole corn (yellow)	33 ,,
Wheat gluten, powdered ...	15 ,,
Gelatin, powdered	15 ,,
Calcium carbonate	3 ,,
Sodium chloride	1 part

The wheat was a mixture of American and Danish wheat (Havnemöllen Ltd., Copenhagen). The yellow corn was La Plata corn (Ballerup Mill Ltd., Copenhagen). The wheat gluten was prepared from a mixture of American and Danish wheat (Leerbeck and Holm, Copenhagen). The gelatin was the

brand V.I. (T. M. Duché and Sons, London). The inorganic salts were chemically pure (*pro analysi*).

All additions of cod-liver oil were given separately in a glass dish as solutions in peanut oil, so that in each case the addition has been 0·20 g.

In this laboratory we prefer to give the addition as mentioned rather than mix it with the food or put it directly into the mouth of the animal. We find it more convenient and just as reliable as the last-mentioned procedure, as the animals devour it completely at once.

X-ray examination. When the animals are entered in the tests, X-ray examination of the left tibia is made at the same time, without anaesthesia. By means of a rubber band the animal is fastened in a wooden box in such a manner that the projection is the same in all the exposures. The X-ray examination is carried out with a Wappler X-ray dental apparatus. The time of exposure is $\frac{1}{4}$ second at 15 cm. with a current of 15 milliamperes and a tension at the tube of *ca.* 50 kilovolts. In judging the X-ray findings importance has been attached to the width of the epiphyseal line and to the density of the metaphysis. The changes are indicated by designations from $\div$ to 3+; or, in case of extreme changes 4+. At the end of the test, X-ray examination is again made of the left tibia under the same conditions.

Autopsy. The animals are killed with illuminating gas and autopsy is performed. This examination has aimed at the following points. (1) Whether the costochondral junctions are swollen, whether there are any so-called costal fractures, and whether there is a "Harrison's groove." (2) Whether the epiphyseal line of the proximal end of the left tibia is wider than normal. The normal width is taken as $\frac{1}{4}$ mm. or less [Schulz, 1929]. The greatest width of this epiphyseal line in rachitic rats has been 1–1$\frac{1}{4}$ mm. (3) Whether the bone is soft to the knife. No great importance has been attached to this point, however, on account of the subjective character of its estimation. The degree of the changes revealed by autopsy has been designated by values ranging from $\div$ to 3+, in particularly severe degree of rickets 4+.

Line test. The line test of Shipley has been carried out in the curative experiments. After the upper end of the left tibia has been divided by sagittal section, one of the cut surfaces is immersed in a 1 % silver nitrate solution, and in this solution it is irradiated by sunlight or by a mercury quartz lamp (Alpine Sun, Hanau); with the last source of radiation usually for 40 seconds. Then the cut surface is examined under a lens with 6 × magnification. Under the given experimental conditions the results obtained with this method have been so inconstant as to make their quantitative estimation questionable. Still the relative values have been entered in the Tables, and their significance will be discussed later. In the estimation of these findings, as far as possible, I have followed the scheme of Bills, but only from $\div$ to 3+; in several instances where such an estimation has been impracticable, this is shown in the table by a question mark. In computing the average values these doubtful animals are not taken into account.

Table I. *Preventive method.*

In every case the experimental period has been 28 days. X-ray examination was made of every animal at the beginning of the experiment, when all the rats were found free of rickets. The X-ray findings recorded are the results of the X-ray examination at the conclusion of each test.

Litter	Rat No.	Addition	Initial weight g.	Gain in weight g.	X-ray examination	Autopsy	Bone analysis: Dry substance %	Bone analysis: Ash %
491	1832 ♀	0	69	37	4+	3+	48·8	19·8
Age of mother: 10½ months.	1833 ♂	0	76	68	3+	3+	45·8	19·3
3rd litter	1834 ♂	1 mg. cod-liver oil	72	60	3+	2½+	62·5	24·5
	1835 ♀	1 ,,	64	43	3+	2½+	52·8	25·1
	1836 ♀	3 ,,	71	44	÷	÷	58·4	30·9
574	2098 ♀	0·2 g. peanut oil	46	29	3+	3+	45·0	12·7
Age of mother: 10 months.	2099 ♂	1 mg. cod-liver oil	49	40	2½+	2½+	43·0	14·2
4th litter	2100 ♂	2 ,,	43	39	½+	2+	46·9	17·0
	2101 ♂	2 ,,	47	31	÷	1+	50·8	19·4
	2102 ♀	3 ,,	47	17	÷	÷	59·4	24·5
	2103 ♀	3 ,,	46	25	÷	÷	53·4	22·6
	2104 ♀	5 ,,	43	25	÷	÷	56·9	23·1
	2105 ♀	5 ,,	46	22	÷	÷	54·0	19·7
	2106 ♂	10 ,,	50	31	÷	÷	55·2	23·7
586	2167 ♀	0·2 g. peanut oil	59	37	3+	3+	45·8	19·2
Age of mother: 15 months.	2159 ♂	1 mg. cod-liver oil	56	32	2+	2+	48·4	21·5
7th litter	2161 ♂	1 ,,	61	33	1+	1½+	52·8	23·9
	2160 ♂	2 ,,	59	38	÷	÷	53·8	25·5
	2162 ♀	2 ,,	50	29	÷	÷	53·7	24·9
	2164 ♀	3 ,,	63	40	÷	÷	53·8	25·5
	2165 ♂	3 ,,	53	39	÷	÷	53·6	24·4
	2163 ♀	5 ,,	50	29	÷	÷	56·2	27·8
	2166 ♀	10 ,,	53	42	÷	÷	54·9	28·0
640	2373 ♂	0·2 g. peanut oil	39	32	3+	3+	43·8	15·2
Age of mother: 4 months.	2374 ♀	1 mg. cod-liver oil	42	25	1½+	1½+	45·0	19·8
1st litter	2375 ♀	2 ,,	42	35	½+	½+	51·1	22·3
	2376 ♀	2 ,,	38	34	÷	½+	52·2	23·1
	2377 ♀	3 ,,	38	34	÷	÷	53·0	24·5
	2378 ♀	3 ,,	38	32	÷	÷	54·0	25·0
	2379 ♂	5 ,,	46	36	÷	÷	55·1	26·6
	2380 ♂	10 ,,	38	36	÷	÷	53·4	21·8
641	2381 ♀	0·2 g. peanut oil	46	34	3+	3+	45·3	17·5
Age of mother: 8 months.	2382 ♂	1 mg. cod-liver oil	51	30	2+	2+	48·5	19·8
2nd litter	2383 ♀	1 ,,	48	33	2+	2+	47·8	20·4
	2384 ♀	2 ,,	47	27	÷	1+	51·4	22·9
	2385 ♂	3 ,,	46	33	÷	÷	52·4	22·0
	2386 ♂	5 ,,	48	38	÷	÷	53·4	25·7
	2387 ♀	10 ,,	50	34	÷	÷	54·0	23·7
661	2448 ♂	0·2 g. peanut oil	42	30	3+	3+	44·7	14·8
Age of mother: 9 months.	2447 ♀	1 mg. cod-liver oil	36	26	2½+	2½+	49·0	19·9
3rd litter	2449 ♀	5 ,,	35	27	÷	÷	55·0	24·8
	2451 ♂	5 ,,	39	33	÷	÷	54·7	24·6
	2450 ♀	10 ,,	36	20	÷	÷	54·4	24·7
	2452 ♂	10 ,,	39	30	÷	÷	54·4	23·5
682	2546 ♂	0·2 g. peanut oil	30	28	4+	4+	40·0	12·9
Age of mother: 4 months.	2540 ♀	2 mg. cod-liver oil	32	33	÷	÷	55·2	23·4
1st litter	2541 ♀	5 ,,	29	32	÷	÷	54·7	24·8
	2542 ♀	10 ,,	32	42	÷	÷	52·1	24·5
	2543 ♀	10 ,,	30	34	÷	÷	54·1	23·0
	2544 ♀	20 ,,	30	43	÷	÷	54·1	25·6
	2545 ♀	20 ,,	29	39	÷	÷	52·4	23·9
689	2563 ♂	0·2 g. peanut oil	38	44	4+	4+	38·9	13·2
Age of mother: 7½ months.	2564 ♂	2 mg. cod-liver oil	37	37	3+	3+	44·6	17·8
3rd litter	2565 ♂	5 ,,	42	49	÷	÷	51·6	24·2
	2566 ♂	10 ,,	41	49	÷	÷	50·0	21·7
	2567 ♀	20 ,,	36	39	÷	÷	50·5	24·2
	2568 ♂	20 ,,	42	38	÷	÷	52·3	23·9
	2569 ♂	40 ,,	39	46	÷	÷	51·7	25·0
	2570 ♂	40 ,,	40	55	÷	÷	51·6	23·8

Table II. *Curative method.*

The fore-period of every litter has been 28 days, except litter 533 (26 days) and litter 600 (31 days). The after-period of every litter has been 7 days. R I means the X-ray examination at the beginning of the after-period, R II at its conclusion.

Litter	Rat No.	Addition	Initial weight g.	Weight increase: Fore-period g.	Weight increase: After-period g	R I	R II	Autopsy	Line test	Dry substance %	Ash %
524	1945 ♂	0	49	35	6	3+	3+	3+	÷	46·8	16·4
Age of mother:	1946 ♂	0	48	37	11	3+	3+	3+	÷	48·8	17·4
6½ months.	1947 ♂	5 mg. cod-liver oil	46	38	12	3+	2½+	3+	2+	46·5	17·7
3rd litter	1948 ♂	5 ,,	49	43	6	3+	2+	2+	3+	46·8	17·2
	1949 ♂	10 ,,	50	41	7	3+	2+	2+	3+	47·4	18·1
	1950 ♂	10 ,,	53	41	13	3+	2+	2+	3+	45·5	14·4
	1951 ♀	20 ,,	51	31	11	3+	½+	1+	3+	51·7	22·0
	1952 ♂	20 ,,	51	36	6	2+	½+	½+	3+	50·5	20·5
533	1962 ♀	0	62	39	÷2	2+	3+	3+	?	55·3	23·8
Age of mother:	1963 ♂	0	69	42	3	2+	3+	3+	÷	50·5	18·0
14 months.	1966 ♀	20 mg. cod-liver oil	59	38	2	3+	2+	1+	3+	53·2	21·8
6th litter	1965 ♂	20 ,,	63	46	6	2½+	½+	2+	?	52·6	22·9
	1964 ♂	40 ,,	62	43	÷4	3+	÷	½+	?	52·8	19·9
	1967 ♀	40 ,,	54	38	÷2	2+	÷	÷	3+	56·2	24·0
542	1991 ♂	0	58	45	6	3+	3+	3+	÷	45·1	13·3
Age of mother:	1992 ♀	0	51	42	5	2½+	2½+	2½+	?	49·8	20·4
7½ months.	1993 ♀	0	52	34	2	2+	2+	3+	÷	51·3	20·4
3rd litter	1994 ♂	10 mg. cod-liver oil	56	48	8	2+	1+	2½+	1+	52·0	19·4
	1995 ♂	20 ,,	54	45	4	3+	1½+	2+	2+	49·7	19·7
	1996 ♂	40 ,,	55	42	0	3+	1½+	2+	1+	48·8	17·7
	1997 ♂	40 ,,	52	42	7	3+	÷	1½+	3+	50·8	17·1
555	2052 ♀	0	50	25	5	3+	3+	3+	÷	49·8	17·7
Age of mother:	2053 ♂	10 mg. cod-liver oil	52	40	3	3+	1+	2+	2+	48·6	18·0
16 months.	2054 ♂	10 ,,	50	37	3	3+	½+	1+	½+	53·5	18·7
7th litter	2055 ♂	20 ,,	52	38	1	3+	½+	2+	2+	50·7	16·2
	2056 ♀	20 ,,	48	36	1	3+	1½+	2+	1+	50·9	21·3
	2057 ♂	40 ,,	51	36	2	3+	2+	1+	1+	49·8	19·1
566	2094 ♂	0·2 g. peanut oil	50	24	9	3+	3+	3+	÷	46·3	15·0
Age of mother:	2092 ♂	10 mg. cod-liver oil	50	32	5	3+	2+	2+	3+	49·1	20·0
8 months.	2093 ♀	10 ,,	45	33	3	3+	2+	3+	2+	45·9	18·7
4th litter	2091 ♂	20 ,,	47	37	6	3+	2+	2+	3+	44·3	17·8
	2095 ♀	20 ,,	47	30	3	3+	1+	1½+	3+	49·3	19·5
	2096 ♂	40 ,,	49	42	6	3+	2+	2½+	2+	42·8	16·2
	2097 ♂	40 ,,	49	39	3	3+	1½+	2½+	?	45·8	16·0
600	2230 ♂	0·2 g. peanut oil	50	45	8	3+	3+	3+	÷	43·3	15·0
Age of mother:	2236 ♀	10 mg. cod-liver oil	46	44	8	3+	2½+	3+	?	43·6	17·8
6½ months.	2235 ♂	10 ,,	48	46	8	3+	1+	1½+	?	46·8	19·6
2nd litter	2234 ♀	20 ,,	42	41	8	3+	1½+	½+	3+	50·2	23·6
	2233 ♀	20 ,,	52	41	10	3+	1½+	1+	3+	47·7	20·7
	2232 ♀	40 ,,	48	37	10	3+	½+	1+	3+	51·5	23·4
	2231 ♀	40 ,,	46	38	11	3+	÷	÷	3+	50·3	24·7
617	2282 ♂	0·2 g. peanut oil	43	27	3	2½+	2½+	3+	÷	43·9	15·2
Age of mother:	2283 ♀	10 mg. cod-liver oil	42	31	2	2½+	½+	1+	1+	46·1	18·5
11 months.	2284 ♀	10 ,,	42	28	4	3+	1½+	2+	1+	45·4	18·1
4th litter	2285 ♀	20 ,,	47	30	0	3+	2+	3+	2+	42·0	15·2
	2286 ♂	20 ,,	48	37	1	3+	1+	2+	3+	44·4	17·0
	2287 ♂	40 ,,	46	30	4	3+	÷	1+	2+	45·7	18·1
	2288 ♂	40 ,,	48	25	6	3+	1+	3+	2+	43·9	15·8

Analysis of the bones. At conclusion of the autopsy, the right tibia is dissected out rapidly. The greatest care has been employed in every instance to prepare the bone in the same way. The periosteum, menisci and ligaments are removed. The fibula is always cut off in the same place. The weighing of the bone is done as quickly as possible and the bone is then placed in an electrical drying-oven, where it is left for 72 hours at 100°. Such an intensive drying process is required, in order to ascertain the dry substance content of the bone. After 72 hours, the bones are constant in weight.

The incineration is done in a muffle-oven heated by 4 Méker burners. The temperature corresponds with that of light red heat. In some experiments with the purest $CaCO_3$ from Kahlbaum, it was ascertained that this temperature was sufficient to calcine this substance into CaO in less than $\frac{1}{4}$ hour. The bone is placed in the oven in a small porcelain crucible and calcined for $\frac{1}{4}$ hour. It is then taken out of the oven, cooled and moistened with 2 drops of concentrated nitric acid, after which it is again heated in the oven to a red heat for 10 minutes. In every instance this procedure has yielded a firm snow-white ash which does not lose in weight on further heating. After cooling in the desiccator, the final weight is recorded. The results of these examinations are given in detail in Tables I and II and are summarised in Tables III and IV, arranged according to the nature of the addition to the diet. Thus, each group comprises all the rats on the respective addition, giving the limits of variation of the dry substance percentage of the bones, ash percentage, X-ray findings, autopsy findings, and line test. The tables give further the average values obtained in these examinations, but under the last three headings the averages are entered in parentheses, as, I think, no great significance is attributable to these averages.

Table III. *Results obtained by the preventive method (Table I) arranged according to dose of addition.*

V means Extent of variation. A means Average.

Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy
Peanut oil or no addition	9	V: 38·9/48·8 A: 44·3	V: 12·7/19·8 A: 16·1	V: 3+/4+ (A: 3·3+)	V: 3+/4+ (A: 3·2+)
1 mg. cod-liver oil	9	V: 43·0/62·5 A: 49·5	V: 14·2/25·1 A: 20·6	V: 1+/3+ (A: 2·3+)	V: 1½+/3+ (A: 2·2+)
2 „	9	V: 46·9/55·2 A: 51·9	V: 17·0/25·5 A: 22·3	V: ÷/½+ (A: 0·1+)	V: ÷/2+ (A: 0·6+)
3 „	8	V: 52·4/59·4 A: 54·8	V: 22·0/30·9 A: 24·9	V: 0 (A: ÷)	V: 0 (A: ÷)
5 „	9	V: 51·6/56·9 A: 54·7	V: 19·7/27·8 A: 24·6	V: 0 (A: ÷)	V: 0 (A: ÷)
10 „	9	V: 50·0/57·4 A: 54·0	V: 21·7/28·0 A: 23·8	V: 0 (A: ÷)	V: 0 (A: ÷)
20 „	4	V: 50·5/54·1 A: 52·3	V: 23·9/25·6 A: 24·4	V: 0 (A: ÷)	V: 0 (A: ÷)
40 „	2	V: 51·6/51·7 A: 51·7	V: 23·8/25·0 A: 24·4	V: 0 (A: ÷)	V: 0 (A: ÷)

Table III shows a gradual rise of the averages with increase of the dose up to 3 mg. cod-liver oil, which is thus the preventive dose. The X-ray and the autopsy findings run very nearly parallel, falling off in + values up to the 3 mg. dose, at which both the X-ray examination and the autopsy give invariably negative results. From percentage of dry substance and of ash, X-ray and autopsy findings, it is consistently evident that cod-liver oil in doses above 3 mg. has had no additional effect. If, with this information in

mind, one turns back to Table I and surveys the material, litter by litter, it becomes strikingly obvious that practically every one of the litters that had a suitable dose of cod-liver oil allows of the same conclusion with regard to the size of the preventive dose. This applies in particular to the X-ray and the autopsy findings. This point is illustrated especially by the litters 574, 640, 661, 682 and 689, whereas litters 586 and 641 leave some doubt whether 3 mg. cod-liver oil has really produced the greatest possible effect on the dry substance and ash content of the bone. In these two litters, however, the X-ray and autopsy findings are in keeping with the findings in the other litters.

Table IV. *Results obtained by the curative method (Table II) arranged according to dose of addition.*

V means Extent of variation. A means Average.

Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy	Line test
Peanut oil or no addition	11	V: 43·3/55·3 A: 48·3	V: 13·3/23·8 A: 17·5	V: 2½+/3+ (A: 2·8+)	V: 2½+/3+ (A: 3·0+)	V: 0 (A: ÷)
5 mg. cod-liver oil	2	V: 46·5/46·8 A: 46·7	V: 17·2/17·7 A: 17·5	V: 2+/2½+ (A: 2·3+)	V: 2+/3+ (A: 2·5+)	V: 2+/3+ (A: 2·5+)
10 mg. cod-liver oil	11	V: 43·6/53·5 A: 47·6	V: 14·4/20·0 A: 18·3	V: ½+/2+ (A: 1·5+)	V: 1+/3+ (A: 2·0+)	V: ½+/3+ (A: 1·8+)
20 mg. cod-liver oil	13	V: 42·0/53·2 A: 49·0	V: 15·2/23·6 A: 21·4	V: ½+/2+ (A: 1·2+)	V: ½+/3+ (A: 1·6+)	V: 1+/3+ (A: 2·6+)
40 mg. cod-liver oil	11	V: 42·8/56·2 A: 48·9	V: 15·8/24·7 A: 19·3	V: ÷/2+ (A: 0·8+)	V: ÷/3+ (A: 1·4+)	V: 1+/3+ (A: 2·2+)

Table IV tells a rather different story. From the averages it will be noticed that the curative dose is about 20 mg. cod-liver oil. The averages of the percentage dry substance and ash show that the outcome of the test falls considerably short of complete recovery, these averages corresponding approximately with the results obtained with the 1 mg. dose in the preventive method. Besides, the difference of the averages is not so distinct by far as that in Table I, and the significance of the average values loses in weight on account of the wide range of the variations; moreover, the limits of variation for the different doses overlap to such an extent that they do not by themselves give any distinction between large and small doses. This is decidedly in contrast with the findings under the preventive method. With the preventive method the lower level of variation shows a gradual rise till the preventive dose is reached, after which this limit does not change to any significant degree.

In other words an attempt to estimate the curative dose by considering a single litter would be impossible; it would be necessary to work with the averages for several litters.

The X-ray and autopsy findings leave a similar impression. The average values of these examinations convey in all essentials the same impression as do the bone analyses; yet it should be pointed out that in these examinations

the lower limit of variation (corresponding to the highest degree of recovery) gives more exact information than does the corresponding limit in the bone analyses. Judging from this lower limit, 40 mg. cod-liver oil would give a greater effect than 20 mg., but I do not think that one may draw any conclusions as to this point from the relatively few animals, especially when the upper limit of variation comes as high as it does in these tests.

Of the line test, really, there is only this to be said, that it turned out negative in the negative control animals and positive in all the cases where cod-liver oil was given, leaving out those cases where the estimation was difficult on account of irregularities. The extent of variation and the average values do not allow of any conclusions.

Discussion.

It seems to me that these experiments suggest that in estimation of vitamin D the preventive method is preferable to the curative method. The experimental period is shorter, and there is less variation in the results from animals on the same addition. The latter point means that the estimation may be carried out with a smaller number of animals. In their employment, upon the whole, the preventive method is the more simple of the two. Here the point is to ascertain whether the test animal has rickets or not—which is comparatively easy with the criteria employed in this investigation. In the curative method the degree of rickets is the important point; and, as is evident from these experiments, this is not easy to estimate. One difficulty is involved in the relatively wide variation of the results in test animals on the same addition; in the bone analyses one may be somewhat justified in judging by an average value. The other difficulty arises if one wants an average estimate on the basis of the X-ray and autopsy findings together with the line test. In relying on an average of one's arbitrary gradations, it is prerequisite that these gradations express the degree of rickets in a certain regular progression—a presupposition that is naturally uncertain. The less arithmetic employed in these biological tests, the safer is the method.

If the question is raised whether the preventive method requires a comparison of the bone analyses, the X-ray examinations and the autopsy findings, in order to settle the presence or absence of rickets, a review of Table I will show that the X-ray examination and the autopsy have separately given the same result as the bone analysis.

Summary.

1. A precise description is given of the technique employed in the preventive and the curative methods for estimation of vitamin D. The results are judged by bone analysis, X-ray examination and autopsy.

2. Parallel series of experiments are made by the preventive and curative methods; the same cod-liver oil is used in both series.

3. A comparison of the results shows that the preventive method is superior to the curative, especially because the animals behave more alike in tests with the first method, so that each estimation can be carried out with a smaller number of animals.

4. A review of the records shows that the bone analyses do not give other or more uniform results than are obtained by X-ray examination and autopsy.

5. On the basis of these findings together with some general considerations, the preventive method is recommended as the standard method for estimation of vitamin D. X-ray examination or autopsy gives sufficient information for judging the results of the test.

REFERENCES.

Adams and McCollum (1928). *J. Biol. Chem.* **78**, 495.
Bills, Honeywell, Wirick and Nussmeyer (1931). *J. Biol. Chem.* **90**, 619.
Cavins (1924). *J. Biol. Chem.* **59**, 237.
Coward (1928). *Quart. J. Pharm.* **1**, 27.
Gudjónsson (1930). *Biochem. J.* **24**, 1591.
McCollum, Simmonds, Shipley and Park (1922). *J. Biol. Chem.* **51**, 41.
Poulsson and Løvenskiold (1928). *Biochem. J.* **22**, 135.
Schulz (1929). *Z. Kinderheilk.* **47**, 449.
Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.

CXC. THE BLOOD-SUGAR LEVEL IN VITAMIN B_1 DEFICIENCY.

By MURIEL EMMA BELL[1].

From the Department of Physiology and Biochemistry, University College, London.

(*Received September 1st, 1931.*)

THERE is still some uncertainty about the reducing value of blood in vitamin B deficiency. The recent contributions on this subject include that of Drummond and Marrian [1926], who found a rise in the blood-sugar of rats fed on a diet completely deficient in B-vitamins, and that of Sure and Smith [1929], who seemed to have provided an explanation when they reported that the rise was due to non-sugar reducing substances in the blood of vitamin B_1-deficient rats. Drummond and Marrian had used the original Hagedorn-Jensen method; Sure and Smith applied Folin's ferricyanide reduction method [1928] to the filtrates obtained by using the Folin-Wu tungstate precipitation [1919] and the Somogyi zinc hydroxide precipitation [1928]. In view of further recent work on the nature of the reducing substances of blood, it seemed desirable to re-investigate the problem, and the original idea was to ascertain what non-sugar reducing substance was responsible for the apparent rise in sugar.

As a preliminary to employing the methods at present regarded as orthodox [Cori, 1931] for arriving at "true sugar" and non-sugar reducing values, series of human bloods and of pigeons' bloods were analysed to compare the Somogyi and the Hagedorn-Jensen zinc filtrates. The Hagedorn-Jensen ferricyanide reduction was used throughout; the filtrates used were the Folin-Wu for total reduction, the Somogyi zinc filtrate, and, in pigeons, the unlaked filtrate of Herbert and Bourne [1930, 1] for "true sugar" values.

It has been shown that for normal and pathological human bloods [Herbert and Bourne, 1930, 2], the non-sugar reducing fraction runs parallel with the cell volume, as the greater part of this fraction is contained in the corpuscles. Accordingly, the cell volume was determined by half-an-hour's centrifuging in haematocrit tubes at 1500 revolutions. Sure and Smith's results seemed to indicate that there would be a very large increase in the cell volume or that there must be some entirely new reducing substance present in bloods from vitamin B_1-deficient animals.

In the following series, the bloods both of pigeons and of rats during normal and vitamin B_1-deficient periods, have been studied. Pigeons were studied in the first instance, partly because they could better spare the blood needed for

[1] William Gibson Research Scholar.

repeated bleedings and for duplicate estimations, partly because they have a higher figure than rats both for sugar and for non-sugar reducing substances, as shown by Gulland and Peters [1930]; hence the risk of experimental error of any magnitude, with the micro-methods which must be used, would be lessened.

Experimental.

Comparison of Hagedorn-Jensen with Somogyi zinc filtrates from human blood.

It may seem unnecessary to produce further evidence that the Hagedorn-Jensen filtrate does not give absolutely true sugar values when several workers have already confirmed Somogyi's results [Herbert and Bourne, 1930, 1; Ege and Roche, 1930]; but as the Hagedorn-Jensen method of precipitation is still widely used, and as, for example, Gulland and Peters [1930] state that "zinc filtrates" (unqualified) "do not contain aliphatic —SS— and —SH— compounds," and that "zinc filtrates are the most reliable for estimating reducing substances in avian blood," it seemed advisable to go over this ground once more in order to see whether there were any significant differences between the two methods of zinc hydroxide precipitation. Somogyi claims that adjusting the reaction to neutrality or slight alkalinity not only precipitates the proteins completely, but also removes the non-sugar reducing substances.

In a recent communication, Kramer and Steiner [1931] have raised an objection to the use of cotton-wool plugs for filtering the coagulated proteins on the ground that they retained some of the sugar. Comparing this filtrate with that of Somogyi they obtained values of 20–30 mg. higher for human blood with the former than with the latter; Somogyi's [1930] comparison between these two methods similarly gave differences of 20–30 mg. On the other hand, Herbert and Bourne found values which were only 1–10 mg. higher; Ege and Roche similarly obtained only a 5–15 mg. difference. Ferricyanide reduction was used in all these instances. The discrepancies were explained by Kramer and Steiner by the inadequate washing of the cotton-wool plugs. In a private communication Herbert and Bourne state that they used, not cotton-wool, but 4 cm. filter-papers, and that a third washing did not alter their result. In order to clear up this point, the following procedure was adopted, using a micro-technique and ferricyanide reduction.

1. Somogyi's No. 1 procedure, *i.e.* using 1·8 % $ZnSO_4, 7H_2O$ and 0·1 *N* NaOH. The solutions were adjusted and precipitation effected as specified by Somogyi.

2. It will be observed that the actual amount of zinc sulphate added is four-fifths of that used in the Hagedorn-Jensen filtrate. (It may be remarked in passing that Hagedorn and Jensen do not state specifically in their original paper that it is the $ZnSO_4, 7H_2O$ which is to be used, but it is apparently the common practice to use the salt containing water of crystallisation.) Therefore, in order to make the two methods comparable in all respects except in the amount of zinc sulphate added, the Hagedorn-Jensen filtrate was prepared

according to the following directions. To 0·2 cc. blood, laked in 5·5 cc. water add 1·25 cc. of 1·8 % $ZnSO_4, 7H_2O$ and 1·0 cc. of 0·1 *N* NaOH. Place in boiling water-bath for 3 minutes. Filter through filter-paper (starch-free). Take 5 cc. of the filtrate. The procedure was the same, therefore, for the Hagedorn-Jensen as for the Somogyi technique, except that the amount of zinc sulphate was 1·25 times greater in the former than in the latter, and that there had been no application of heat in the latter. The following points were attended to: the amount of fluid in the boiling-tubes was made up to 14 cc.; the tubes were covered during and after reduction to minimise re-oxidation; the ferricyanide was twice recrystallised from a "recrystallised" sample. Care was taken to see that the blood was adequately mixed, to ensure even distribution of the corpuscles.

The figures obtained for human blood are given in Table I. It will be seen that the differences range from 5 to 15 mg. with an average of 8, which compares very closely with those recorded by Herbert and Bourne and by Ege and Roche.

Table I. *Comparison between Somogyi and Hagedorn-Jensen filtrates. Human blood.*

	Sugar value (mg./100 cc.)		
Sample	Somogyi filtrate	Hagedorn-Jensen filtrate	Difference
1	90	105	15
2	116	123	7
3	105	112	7
4	102	109	7
5	90	99	9
6	64	69	5
7	73	86	13
8	88	93	5
9	70	80	10
10	80	86	6
		Average	8

The cotton-wool plugs accordingly do not seem to explain the discrepancy, as suggested by Kramer and Steiner. The latter state that they used 0·30 to 0·35 cc. *N*/10 NaOH to neutralise their filtrate. As it seemed possible that they might perhaps have made their zinc sulphate solution to contain 0·45 % of $ZnSO_4$ instead of 0·45 % $ZnSO_4, 7H_2O$, a few human bloods were tested using the additional zinc sulphate, neutralising and not neutralising the filtrate, and again taking an aliquot part of the filtrate. The effect of the extra zinc sulphate was to increase the difference in reduction between the Somogyi and the second filtrate only if the latter were neutralised. Apparently, not neutralising the filtrate diminished the efficacy of the ferricyanide reagent, with the result that the figures were much the same as the ordinary Hagedorn-Jensen figures.

Herbert and Bourne have shown that the p_H of the Hagedorn-Jensen filtrate is 6·8, while that of the Somogyi filtrate is 7·4. In this investigation, the p_H was similarly determined on a few samples, using the method of Dale and Evans [1920]. The average values thus obtained were 6·85 and 7·2 re-

spectively. It would seem, therefore, that the critical p_H is the neutral point, as Somogyi claims.

Reducing values for pigeons' blood.

Whereas the evidence put forward by several different workers is that the two zinc filtrates give slightly different results for human blood, the difference is more marked when they are applied to avian blood, with its greater content of non-sugar reducing substances. It was stated by Gulland and Peters that 70 mg. in terms of glucose was the average for the non-sugar reducing substances of pigeons' blood. They, however, used the Hagedorn-Jensen filtrate to give total reduction, and blood which has been allowed to undergo slow spontaneous glycolysis to obtain their residual reduction. The Hagedorn-Jensen filtrate, as Table II shows, does not allow through more than a part of

Table II. *Comparison between Hagedorn-Jensen*[1] *and Somogyi filtrates. Pigeons' blood.*

	(mg./100 cc.)			
No.	"Total sugar" (Folin-Wu filtrate)	Somogyi filtrate	Hagedorn-Jensen filtrate	Difference
1	256	192	208	16
2	256	198	230	32
3	227	176	207	31
4	254	198	224	26
5	315	259	291	32
6	334	279	300	21
7	230	163	190	27
8	227	176	207	31

the non-sugars, and it is possible that blood after slow glycolysis has not lost all its glucose. Moreover, in the absence of glucose, as Herbert, Bourne and Groen [1930] have shown, glutathione has a rather higher glucose equivalent. It is suggested, therefore, that the figure of 70 mg. was obtained by a compensation of errors.

Blood was obtained by puncturing the wing vein, the bird being kept quiet by putting its head under the opposite wing. The blood was rapidly mixed with a minimal quantity of oxalate. 0·1 cc. blood was used for an estimation in each case.

Normal birds were fed with poultry feed. The deficient diet offered was polished rice and Kon and Drummond's diet [1927]. When the birds refused to eat the deficient diet, they were forcibly fed with the latter. About 15 g. were placed in the crop each day; this was insufficient to maintain their weights, but ensured that they were not suffering from starvation.

It will be seen (Tables III and IV) that the average non-sugar reducing value for pigeons' blood by these methods is about 60 mg. in terms of glucose. The cell volume averages 49 %. For human blood, the corresponding figures are 20–30 mg. and 41 % respectively [Herbert and Bourne, 1930, 2]. The

[1] Using an aliquot part of the filtrate.

Table III. *Normal pigeons fasting 24 hours.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
"Squeakers"						
734	8. vii. 31	—	130	—	—	—
734	17. vii. 31	206	133	130	73	51
736	8. vii. 31	243	182	—	61	—
736	17. vii. 31	264	202	206	62	—
742	8. vii. 31	206	150	—	56	46
742	17. vii. 31	184	130	133	54	—
478	8. vii. 31	203	136	139	67	51
478	17. vii. 31	224	154	—	70	52
731	8. vii. 31	205	150	150	55	46
731	17. vii. 31	221	154	—	67	—
	Average	217	152	—	63	49
Adult birds						
p.s.r.	19. v. 31	249	181	178	68	49
1707	19. v. 31	243	179	—	64	—
9754	19. v. 31	245	179	181	66	50
1478	19. v. 31	252	194	—	58	45
p.p.r.	20. v. 31	238	179	174	59	50
9738	20. v. 31	229	167	167	62	51
9731	22. v. 31	254	198	198	56	—
9743	22. v. 31	237	176	176	61	47
	Average	243	182	—	62	49

Table IV. *Normal pigeons feeding.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
"Squeakers"						
736	2. vii. 31	246	195	—	51	—
734	2. vii. 31	242	174	—	68	55
731	2. vii. 31	253	184	184	69	47
742	2. vii. 31	235	165	165	70	49
478	2. vii. 31	216	162	—	54	51
	Average	238	176	—	62	50
Adult birds						
9742	25. iv. 31	249	195	192	54	43
375	27. iii. 31	283	229	—	54	—
375	25. iv. 31	293	228	222	65	54
9736	31. iii. 31	246	187	187	59	—
p.p.	31. iii. 31	278	—	—	—	—
9735	7. iv. 31	258	206	—	52	—
1478	7. iv. 31	248	203	200	45	—
m.f.	8. iv. 31	268	208	202	60	—
f.w.	8. iv. 31	246	187	—	59	—
9731	8. iv. 31	253	195	—	58	46
9743	8. iv. 31	264	197	200	67	45
1707	9. iv. 31	254	206	—	48	—
9738	10. iv. 31	277	216	—	61	—
p.w.	17. iv. 31	—	212	213	—	—
p.s.r.	26. iii. 31	256	192	—	64	—
p.w.	26. iii. 31	256	198	—	58	47
	Average	262	204	—	58	47

non-sugar reducing content is consequently higher per unit volume of corpuscles in birds' blood than in human blood.

The Hagedorn-Jensen filtrate (Table II) gives values 16–32 mg. higher than the Somogyi filtrate, but not as high as the Folin-Wu filtrate. When these filtrates were tested by the nitroprusside reaction, the Folin-Wu gave a markedly positive colour, the isotonic and the Somogyi were negative, while the Hagedorn-Jensen gave a colour about half as intense as the Folin-Wu. This lends support to the view that the substance mainly concerned in producing non-sugar reduction is glutathione or other sulphydryl-containing substances.

The "true sugar" averages 152 mg. for young birds fasting 24 hours, 182 mg. for adult birds fasting 24 hours; 176 mg. for young birds feeding, 204 mg. for adult birds feeding. The range of figures is a wide one, namely from 130 mg. to 198 mg. for fasting birds, from 162 mg. to 230 mg. for feeding birds. This wide range is probably to be explained by the varying degrees of excitement that the manipulations produce. From the small group of young birds studied, the impression was gathered that the smaller birds had lower amounts of circulating glucose, perhaps connected with their greater intensity of metabolism and more rapid utilisation of glycogen stores. Gulland and Peters deduced from their average of 135 ± 15 mg. glucose that "the true sugar value of birds' blood is not so widely divergent from that of mammals as the Hagedorn-Jensen estimations seem to show." With the methods used here, the figures are higher, in spite of the fact that their method of collecting the sample—by guillotining the bird—is likely to lead to a rise in blood-sugar. One is of the opinion, therefore, that their figures for true sugar are definitely low.

The two methods for true sugar were undertaken, firstly in order to see what effect the extra amount of uric acid contained in birds' blood had on the unlaked filtrate of Herbert and Bourne. Their filtrate is practically identical with that simultaneously proposed by Folin [1930]. It is unlikely that the uric acid would be confined to the corpuscles, and yet the figures show rather surprising agreement between the unlaked and the Somogyi filtrate. The figures given by Folin and Denis [1913] for uric acid in birds' blood are 4·8 mg.; those given by Scheunert and Pelchrzim [1923] are about 2 mg. The reducing value of uric acid, using ferricyanide reduction, is 53 % that of glucose [Hagedorn and Jensen, 1923, 2; Holden, 1926], so that the maximum difference due to uric acid would be 2·4 mg. in terms of glucose, even if it all diffused out of the corpuscle in the time allowed. The second reason for using the unlaked filtrate was to see whether the alleged rise in non-sugar reducing substances in vitamin B_1-deficiency were due to a constituent of the cells or of the plasma; if it were a non-diffusible substance present in the cells, then the isotonic filtrate would show a smaller value than the Somogyi filtrate. It was found, as later results indicate, that the two filtrates gave very similar results during the stage of vitamin B_1-deficiency in pigeons.

Table V. *Vitamin B_1-deficient pigeons during convulsions.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
Adult birds						
p.s.r.	13. iv. 31	507	461	—	46	37
	14. iv. 31	392	336	336	66	35
9754	18. iv. 31	264	197	198	66	43
	19. iv. 31	314	—	258	56	46
	20. iv. 31	382	312	312	70	44
	21. iv. 31	333	—	—	—	—
p.p.	18. iv. 31	340	282	288	58	39
	19. iv. 31	370	314	325	56	37
	20. iv. 31	298	—	261	37	35
9738	22. iv. 31	298	235	228	63	—
f.w.	22. iv. 31	320	275	273	45	38
	23. iv. 31	480	438	419	42	—
	24. iv. 31	—	509	—	—	—
m.f.	23. iv. 31	344	296	296	48	—
	24. iv. 31	299	256	267	43	—
1478	27. iv. 31	406	330	—	76	—
	28. iv. 31	296	232	—	64	—
	29. iv. 31	283	243	245	40	35
9743	28. iv. 31	296	248	251	48	40
	29. iv. 31	259	221	230	38	37
1707	1. v. 31	—	237	243	—	40
	2. v. 31	279	229	235	50	—
9731	8. v. 31	242	197	—	45	32
p.w.	15. v. 31	374	317	306	57	44
9736	23. v. 31	338	253	—	85	48
p.s.	11. vi. 31	262	219	222	43	40
	12. vi. 31	355	296	—	59	—
	13. vi. 31	—	283	—	—	—
	14. vi. 31	315	232	—	83	42
9731	15. vi. 31	—	295	—	—	—
9734	15. vi. 31	340	275	—	65	—
9742	17. vi. 31	229	155	156	74	51
	17. vi. 31	248	190	—	58	—
	18. vi. 31	289	—	228	61	53
	18. vi. 31	412	350	—	62	52
	19. vi. 31	459	398	396	61	52
p.w.	18. vi. 31	515	434	440	75	41
	18. vi. 31	506	459	—	47	—
	19. vi. 31	367	314	—	53	45
9743	19. vi. 31	—	248	251	—	39
p.p.	20. vi. 31	278	—	224	54	48
	20. vi. 31	314	267	—	47	47
1478	20. vi. 31	294	249	—	45	44
9736	21. vi. 31	—	262	—	—	—
	Average	340	290	—	53	41
"Squeakers"						
478	30. vii. 31	334	—	279	55	42
	31. vii. 31	302	253	—	49	43
	1. viii. 31	302	256	—	46	40
	2. viii. 31	—	253	—	—	40
	3. viii. 31	—	267	—	—	—
731	31. viii. 31	534	499	—	—	44
	1. viii. 31	397	336	—	61	—
742	5. viii. 31	251	195	—	56	—
	6. viii. 31	235	165	—	70	60
	6. viii. 31	236	—	151	85	65
734	6. viii. 31	211	170	—	41	—
	7. viii. 31	—	224	—	—	—
	Average	311	272	—	59	49

Blood-sugar changes in pigeons during vitamin B_1-deficiency.

The changes that occurred in "true sugar" during vitamin B_1-deficiency can be seen in Tables V, VI, VII and Fig. 1. During the stage of convulsions, the "true sugar" went up—parallel with the "total sugar"—to an average value of 290 mg. for adult birds and 272 mg. for the group of young birds.

Table VI. *"True sugar" in blood of pigeons during pre-convulsive stage.*

Bird	Date	"True sugar" (Somogyi filtrate) mg./100 cc.	Bird	Date	"True sugar" (Somogyi filtrate) mg./100 cc.
734	4. viii. 31	144	9735	12. v. 31	163
742	4. viii. 31	190	375	12. v. 31	190
734	31. vii. 31	154	p.w.	11. v. 31	182
731	31. vii. 31	224	9731	7. v. 31	218
742	31. vii. 31	176	p.p.r.	16. iv. 31	179
1478	16. vi. 31	173	9738	16. iv. 31	172

Table VII. *"True sugar" in blood from heart of pigeons, dead from vitamin B_1 deficiency.*

No. 742, at death, following convulsions ... 151 mg.
„ 9744, „ (from ascites) ... ... 312 mg.

Blood from heart after death:

No.	478	... ...	90 mg.
„	731	... ...	not less than 256 mg.
„	9731	... ...	27 mg.
„	9734	... ...	275 mg.
„	p.s.r.	... ...	128 mg.
„	734	... ...	378 mg. (dead in position of head retraction)

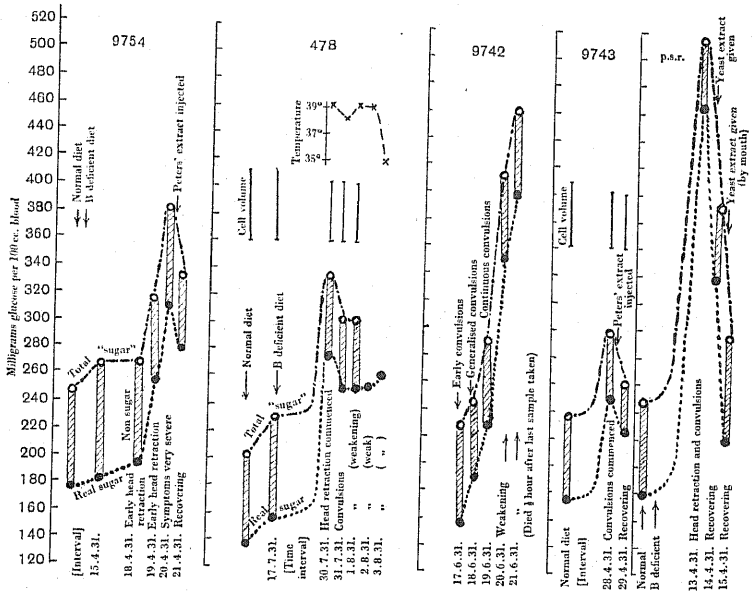


Fig. 1. Graphs of increased reduction in blood of pigeons during convulsions.

Occasionally the "true sugar" reached a figure of 500 mg. Of the twenty birds used, most of which were studied through two periods of deficiency, there was no exception to the finding that a given individual bird will show an increase over its fasting level for sugar, and usually over its feeding level also. The response varied considerably with different individual birds.

The non-sugar reducing substances suffered no appreciable alteration. Occasional high values were attributed to experimental error. The average value was 53 mg., in terms of glucose, for one group of animals, a rather lower figure than the normal average. In this group, furthermore, the average cell volume diminished slightly. Some birds showed a progressive anaemia—and the non-sugar reducing substances followed the cell volume downwards. This is consistent with the finding that the greater part of the non-sugar reducing substances resides in the corpuscles. Occasionally, on the other hand, a bird which developed ascites showed an increase both in cell-volume and in non-sugar reducing substances. Bird 742 is an extreme example of this, where the cell volume at the time of death was 65 %, and the non-sugar value was 85 mg. This was, however, exceptional. The bird had lost water not only into its tissues, but also by the rapid respiration, presumably due to hydropericardium, which it had been experiencing for several hours before death.

Occasionally a bird which did not develop convulsions had a high "true sugar" level, *e.g.* bird 9744 died during manipulations for taking the blood sample. It was found to have ascites, with fluid in the pericardial cavity. Its "true sugar" was at the high level of 342 mg. Undoubtedly an asphyxial element would enter into the production of hyperglycaemia in this case. Only twice was it possible to get blood from the heart immediately after death from convulsions. No. 742 whose temperature was 26° at death had a "true blood-sugar" of 150 mg.; No. 9742 died half-an-hour after the last sample containing 390 mg. had been taken. Even among the birds which had been dead for several hours before their heart-blood was collected, there were three with figures of 256, 275, and 378 mg. respectively. The lower figures of 90 and 27 mg. may have resulted from glycolysis, but may also have been similar findings to those of Drummond and Marrian who frequently found low values at the time of death.

On the whole, in this series, the incidence of hyperglycaemia has been associated with the convulsive stage. It was seldom that any rise occurred in the pre-convulsive stage (Table VI). Extraordinarily high figures were reached in some cases where convulsions were present. A sub-lethal dose of cyanide was accordingly administered to two pigeons to ascertain the effect of convulsions produced in this way. The first developed typical convulsions and gave an increase in "true sugar" from 131 to 259 mg. The second, dosed on two occasions, did not become convulsed, and showed a diminution from 139 to 128 mg. and from 160 to 149 mg. The hyperglycaemia in the first pigeon would no doubt come under the heading of an asphyxial rise in sugar [Macleod, 1926].

An attempt to prove that the raised blood-sugar was due to an increased output of adrenaline was unsuccessful.

Sugar values for rats' blood.

Since vitamin B_1-deficient pigeons had shown no rise in non-sugar reducing substances but were definitely hyperglycaemic during the convulsive stage, rats were now used as the experimental animals.

Normal values were first established and were found to lie, as Sure and Smith stated, within the values of 17–34, with an average of 29 mg. per 100 cc. of blood, in terms of glucose. In the first estimations, a number of (feeding) stock rats were used; they were killed with chloroform, and blood was taken from the heart. A few (feeding) vitamin B-deficient rats were similarly used. For the great majority of the figures, however, blood was taken from the lateral vein of the tail of animals fasting 6 hours or longer. From 0·2 to 0·3 cc. blood was collected with a pipette into a minimal quantity of oxalate.

The vitamin B_1-deficient animals were fed on a diet consisting of rice starch 75 %, caseinogen 20 %, salt mixture 5 %. A supplementary ration of 1 drop of cod-liver oil, and of autoclaved marmite (to supply vitamin B_2) was fed to all the rats; the controls received an additional ration of Peters's extract (vitamin B_1). The animals fasted for at least 6 hours before the sample was taken.

Normal values.

(*a*) "Total sugar"—usually below 120 and above 80 mg., with an average of 109 mg.

(*b*) "True sugar"—usually below 90 and above 50 mg., with an average of 76 mg.

(*c*) Cell volume, average value 44 %.

(*d*) Non-sugar reducing substances, average 29 mg. in terms of glucose.

The vitamin B-deficient animals were classified into those that had convulsions, those that did not develop convulsions, and those that were moribund when the sample was taken (Table VIII). The average cell volume for these groups was 44, 46, and 44 respectively. No consistent alteration occurred in the cell volume. The non-sugar reducing substance averaged 32, 28 and 27 mg. respectively, and in no case was it over 35 mg. Care was taken, when it was suspected that the "real sugar" might be low, to increase the quantity of blood taken for the Somogyi estimation from 0·1 to 0·15 cc. or more if it could be obtained. Duggan and Scott [1926] and Kramer and Steiner [1931] have thrown doubt on the accuracy of estimations of amounts below 25 mg. If that is the case, then a Somogyi estimation, since it is done on five-eighths of the filtrate, must be over 40 mg. to be accurate.

While, contrary to Sure and Smith's finding, no increase was found in the non-sugar reducing value of the blood of vitamin B-deficient rats, on the other hand, in agreement with Sure and Smith, no increase in the "true sugar" was

Table VIII. *Vitamin B-deficient rats, fasting 6 hours.*

No.	Date	"Total sugar"	"True sugar"	Non-sugar reducing substances	Cell volume
		(Convulsions.)			
124	9. vii. 31	71	41	30	44
49	9. vii. 31	123	91	32	43
50	9. vii. 31	63	28	35	—
121	9. vii. 31	96	68	28	—
941	12. vii. 31	—	83	—	—
48	14. vii. 31	111	77	34	—
119	18. vii. 31	101	72	29	45
125	20. vii. 31	112	79	33	—
	Average	97	67	32	44
		(No convulsions. Hunched.)			
43	29. vi. 31	75	53	22	—
40	2. vii. 31	98	75	23	—
122	2. vii. 31	117	100	17	42
117	11. vii. 31	143	118	25	44
126	5. viii. 31	91	74	17	—
128	7. viii. 31	110	78	32	48
125	7. viii. 31	103	74	29	48
126	7. viii. 31	99	64	35	48
43	7. viii. 31	112	83	29	42
46	7. viii. 31	100	70	30	—
50	10. viii. 31	96	—	—	45
49	10. viii. 31	120	96	24	41
129	12. viii. 31	90	64	26	—
133	12. viii. 31	86	54	32	47
132	12. viii. 31	83	54	29	46
131	12. viii. 31	96	72	24	—
130	12. viii. 31	102	68	34	—
132	19. viii. 31	82	56	26	—
51	20. viii. 31	101	72	29	—
39	18. vi. 31	106	74	32	46
40	18. vi. 31	102	70	32	45
	Average	101	73	28	46
		(Moribund.)			
52	29. vi. 31	120	90	30	42
42	2. viii. 31	130	101	29	—
123	10. vii. 31	67	38	29	—
122	10. vii. 31	—	7	—	—
118	10. vii. 31	40	15	25	46
119	20. vii. 31	25	3	22	—
	Average	77	42	27	44

observed. Not even during the stage of convulsions did the rats show a hyperglycaemia. Then began a process of elimination to account for the discrepancy between the findings in this series and the finding by Drummond and Marrian —that a "hyperglycaemia occurs in rats during the greater part of the time that they are on a vitamin B-deficient diet." Firstly, the Hagedorn-Jensen method was too little different from the Somogyi method to account for the discrepancy. Secondly, the diet given by Drummond and Marrian was deficient in B-complex; therefore a few rats deficient in vitamin B_2 only were used, to see whether this was the factor responsible, with the result that they also showed the same normal sugar values. Thirdly, as it was found that Drummond and Marrian had used blood taken from the heart immediately after rapidly

Table IX. *"True sugar" values for rats' blood, showing effect of method by which blood is collected.*

(Rats fasting overnight.) "True sugar" values (Somogyi filtrate).

	mg./100 cc. blood		
Normals No.	Blood from tail	Blood from heart, immediately after rapid death by dislocation of the neck	Rise produced by latter procedure
168	82	112	30
169	74	107	33
173	52	52	0
175	102	106	4
166	106	129	23
170	102	171	69
134	89	96	7
135	85	152	67
172	96	103	7
171	79	126	47
165	70	106	36
1	71	120	49
2	83	96	13
3	68	129	61
4	58	86	28
5	78	96	18
Vitamin B_1-deficient:			
132	56	79	28
125	79	118	39
51	62	79	17
133	48	82	34
130	40	49	9
49	81	139	58

killing the animals by dislocating the neck, a few vitamin B_1-deficient animals were treated in this way. Blood was first taken from the tail, the animals were rapidly killed and blood was taken immediately from the right side of the heart. These latter samples gave higher values than the tail-blood samples. In a series of normal rats treated in the same way, a similar rise was observed in the majority of the second samples. Sometimes there was no appreciable rise. The increase ranged from 0 to 69 mg. (Table IX). This means that it is possible to have something like a 100 % error if blood is collected by this method for sugar estimations. Apparently this fact has not been generally recognised hitherto. The rise occurs with extraordinary rapidity. The physiological explanation is either that of intense excitement before death, or direct stimulation of the spinal cord by the odontoid process of the second cervical vertebra as it penetrates the spinal cord; adrenaline secretion will occur and glycogen be mobilised. As evidence of adrenaline secretion, the blood clotted very rapidly.

No explanation, therefore, has been found for the definitely lower values for blood-sugar found by Drummond and Marrian to occur in the blood of normal rats as compared with vitamin B-deficient rats and at present it appears to be due to coincidence. It will be seen by reference to Table IX, that there is room for that coincidence to have occurred.

Discussion.

These results seem to explain why a hyperglycaemia in pigeons has not always been found. Funk and Schönborn [1914] and several other workers found a rise, but Kon and Drummond [1927] reported no difference in the sugar levels of the two groups of animals which they studied.

It appears to depend upon the stage at which the blood-sample is taken, the hyperglycaemia being definitely associated with the appearance of convulsions. As to the mechanism which produces the rise in blood-sugar, there is no satisfactory evidence. The most probable supposition is that a nervous stimulation occurs, producing adrenaline secretion and consequent mobilisation of glycogen. The absence of a detectable increase in blood-glucose in the rat during convulsions is probably to be explained by depletion of its carbohydrate stores. Kinnersley and Peters [1930] have recently reported finding a local production of lactic acid in the brain of vitamin B-deficient pigeons. If that lactic acid is a cause and not an effect of local stimulation, it may conceivably also be the cause of a *piqûre* type of effect giving rise to adrenaline secretion.

Finally, the author is not of the opinion that the finding of the hyperglycaemia throws any light on the essential nature of vitamin B_1-deficiency. It appears to be a subsidiary effect; the birds are definitely not well before they show the hyperglycaemia; the cause of failure to grow and to maintain health is not explained by it.

Summary.

1. A comparison between the Hagedorn-Jensen and the Somogyi methods of zinc hydroxide precipitation in human blood gave an average value of 8 mg. higher reduction with the former than with the latter.

2. A similar comparison on pigeons' blood gave values 16–32 mg. higher with the former than with the latter.

3. Using Folin-Wu, Somogyi and Herbert and Bourne filtrates, "total sugar," "true sugar," and non-sugar reducing values for fasting, adult pigeons' blood have averaged respectively 243, 182, 62 mg.

4. The isotonic filtrate of Herbert and Bourne gives practically the same values as the Somogyi filtrate for birds' blood.

5. There is a marked rise in "true sugar" during the convulsive stage in vitamin B_1-deficient pigeons.

6. No increase was found in the non-sugar reducing substances or cell volume of the blood of vitamin B_1-deficient pigeons or rats.

7. No increase was found in the "true sugar" of the blood of vitamin B_1-deficient rats.

8. There is usually a fall in blood-sugar in the final stages of vitamin B_1 deficiency in rats, but pigeons dying from convulsions or from hydropericardium are often hyperglycaemic.

9. When blood is obtained from the hearts of rats which have been rapidly killed by dislocation of the neck, it shows an increased sugar content in most

cases compared with circulating blood obtained immediately beforehand from the tail.

10. The hyperglycaemia in pigeons is thought to be the result of nervous stimulation—something of the nature of the hyperglycaemia of asphyxia, anaesthesia or diabetic *piqûre*. Its failure to occur in convulsive rats is ascribed to exhaustion of glycogen stores.

I am indebted to the Royal Society of Medicine for the scholarship which has enabled me to carry out this research, and to the Medical Research Council for a grant to defray the cost of materials.

I wish to thank Prof. Drummond for helpful advice and criticism.

REFERENCES.

Cori (1931). *Physiol. Reviews*, **11**, 147.
Dale and Evans (1920). *J. Physiol.* **54**, 167.
Drummond and Marrian (1926). *Biochem. J.* **20**, 1245.
Duggan and Scott (1926). *J. Biol. Chem.* **20**, 1229.
Ege and Roche (1930). *Scand. Arch. Physiol.* **59**, 75.
Folin (1928). *J. Biol. Chem.* **77**, 421.
—— (1930). *J. Biol. Chem.* **86**, 173.
—— and Denis (1913). *J. Biol. Chem.* **14**, 29.
—— and Wu (1919). *J. Biol. Chem.* **38**, 106.
Funk and Schönborn (1914). *Z. physiol. Chem.* **89**, 373.
Gulland and Peters (1930). *Biochem. J.* **24**, 91.
Hagedorn and Jensen (1923, 1). *Biochem. Z.* **135**, 46.
—— —— (1923, 2). *Biochem. Z.* **137**, 92.
Herbert and Bourne (1930, 1). *Biochem. J.* **24**, 299.
—— —— (1930, 2). *Biochem. J.* **24**, 1787.
—— —— and Groen (1930). *Biochem. J.* **24**, 291.
Holden (1926). *Biochem. J.* **20**, 263.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
Kramer and Steiner (1931). *Biochem. J.* **25**, 161.
Kon and Drummond (1927). *Biochem. J.* **21**, 633.
Macleod (1926). Carbohydrate metabolism and insulin, p. 234. (Longmans, London.)
Somogyi, M. (1928). *Proc. Soc. Exp. Biol. Med.* **26**, 353.
—— (1930). *J. Biol. Chem.* **86**, 655.
Scheunert and Pelchrzim (1923). *Biochem. Z.* **139**, 17.
Sure and Smith (1929). *J. Biol. Chem.* **84**, 727.

CXCI. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

V. SOME FACTORS DETERMINING THE QUANTITY OF NITROGEN IMMOBILISED DURING DECOMPOSITION.

By ERIC HANNAFORD RICHARDS
and ARTHUR GEOFFREY NORMAN.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

(*Received September 1st, 1931.*)

The decomposition of plant materials in the soil or in compost heaps involves the assimilation of the carbohydrate constituents of the tissues and the conversion of available nitrogen to microbial protein by the organisms concerned. In nature the supply of nitrogen is often a limiting factor in determining the rate and extent of decomposition. Mature plant tissues frequently contain rather a low percentage of nitrogen, and to effect a complete and speedy rot, an additional quantity of available nitrogen has to be supplied. If too much is provided, losses occur, or if too little, the decomposition is incomplete unless an unusually long period of time is allowed. Hutchinson and Richards [1921] were the first to recognise the quantitative importance of nitrogen in decomposition, and they determined the amount necessary for the decomposition of a number of materials. For common straws, for example, they found it to be between 0·7 and 0·8 g. nitrogen per 100 g. original material. This figure they termed the "nitrogen factor." It may be defined as the additional inorganic nitrogen immobilised as organic nitrogen by 100 g. of any material in the process of decomposition. This quantitative relationship forms the basis of Richards and Hutchinson's patents [1924] for the production of "artificial farmyard manure" (Adco), from waste plant materials. The "nitrogen factor" gives the maximum amount of nitrogen which when added to decomposing material will be retained without loss.

Table I gives the "nitrogen factors" for a number of common materials, and indicates the range of variation found.

No basis exists for any prediction of the "nitrogen factor" and it has to be determined by trial in each case. The variations which are observed clearly depend on the differences in composition of the various materials and their relative availabilities to the micro-flora present. Accordingly investigations were undertaken to ascertain the nature of the relationship, if any, between

Table I. *"Nitrogen factors" of some waste plant materials.*

Plant material	"Nitrogen factor"	Loss of dry matter %
Willow peelings	1·33	35·2
Sugar cane trash	1·06	27·9
Oat straw	0·85	53·1
Rye straw	0·71	45·0
Rice straw	0·64	35·2
Esparto grass waste	0·52	25·7
Rushes (Scilly Isles)	0·50	21·9
Almond husks	0·44	55·0
Banana leaf	0·42	45·7
Almond skins	0·30	52·2
Flax shives	0·12	19·6

composition and "nitrogen factor." A number of widely different materials, some extracted suitably to remove known constituents, were employed, and rotted under optimum and parallel conditions.

Experimental.

The materials were in each case cut to a suitable size for rotting, about that of chaffed straw. Equal amounts of each, of known moisture content, were taken and moistened thoroughly by spraying. Available nitrogen in the form of ammonium carbonate was added to the material when bottled, together with an inoculum from soil in which organic matter was rotting. The moisture content was kept equal amongst the members of each series. In the case of certain extracted materials moistening was carried out with a mineral salt solution, since the process of extraction was likely to remove necessary ions. Washing in many cases had to be very prolonged to remove all traces of the extracting agents.

For the determination of the "nitrogen factor" the following figures were necessary.

1. Original nitrogen content—Kjeldahl.
2. Final nitrogen content—Kjeldahl.
3. Final ammonia content—distillation with MgO.
4. Loss of organic matter in decomposition.

To determine the composition of the materials employed, the following estimations were carried out as described by Norman [1929].

5. Total furfuraldehyde yield.
6. Cellulose (Cross and Bevan).
7. Furfuraldehyde from Cross and Bevan cellulose.
8. Carbon dioxide yield (uronic acid).

The composition and the "nitrogen factor" at various stages were determined for the materials enumerated in Table II.

Willow peelings were also rotted without the addition of nitrogen and this series will be designated D 2.

Table II. *Percentage composition of plant materials employed.*

Series No.	Plant material	C. and B. cellulose	Furfurald. from C. and B. cellulose	CO_2 yield	Total uronic anhydride	Furfurald. from uronic acids	Total furfurald. yield	Furfurald. from pentoses in hemicelluloses	Pentoses in hemicelluloses as anhydroxylose	Xylan associated with C. and B. cellulose	"Pure" cellulose	Lignin	Total N
A	Flax straw	53·40	6·49	1·69	6·76	1·13	12·04	4·42	6·9	10·1	43·3	30·5	0·51
B	Flax shives (retted)	53·57	6·88	1·29	5·16	0·86	12·56	4·82	7·5	10·7	42·9	30·9	0·21
C	Flax fibre	87·50	1·19	0·22	0·88	0·15	1·85	0·51	0·8	1·8	85·7	Nil	0·55
D	Willow peelings	31·16	1·80	2·69	10·76	1·79	7·37	3·78	5·9	2·9	28·3	36·5	1·58
E	Oat straw	48·92	6·61	1·36	5·44	0·91	15·95	7·43	11·5	10·25	38·7	18·5	0·30
F	Oat straw extract with hot water	50·99	6·97	1·56	6·24	1·04	16·59	8·58	13·3	10·8	40·2	—	0·27
G	Oat straw extract with 4 % NaOH	84·07	7·86	0·26	1·04	0·17	9·01	0·98	1·5	12·8	71·3	11·1	0·12
H	Oat straw extract with 2 % HCl	61·98	1·53	0·61	2·44	0·40	2·57	0·64	1·05	2·4	59·6	25·6	0·34
I	Oat straw extract with 4 % alc. NaOH	65·60	8·70	0·80	3·20	0·53	14·08	4·85	7·5	13·5	52·1	8·2	0·11
J	Young barley straw extract with 4 % NaOH	89·10	2·66	0·31	1·24	0·21	3·55	0·68	1·1	4·1	85·0	—	Nil
K	Esparto grass waste	48·07	5·91	—	—	—	12·66	6·8 (approx.)	10·6	9·2	38·9	26·7	0·95
L	Esparto grass waste extract with 4 % alc. NaOH	55·37	9·45	—	—	—	14·65	5·2 (approx.)	8·1	14·6	40·7	12·3	0·23

The composition of these materials is given in Table II and the "nitrogen factor" determinations in Table III. In addition to the "nitrogen factor," the "nitrogen equivalent" has been calculated. This represents the amount of additional nitrogen immobilised per 100 g. of organic matter lost. The use of this figure as an aggregate measure of efficiency of the organisms has been described in Part IV of this series [Norman, 1931].

In Table II for the sake of comparison the furfuraldehyde from the Cross and Bevan cellulose fraction has been calculated in all cases as xylan. That it is xylan in every case is an unjustifiable assumption, but it has been shown to be so in the case of the flax, the oat straw and the esparto grass.

Discussion of results.

I. "*Nitrogen factor.*"

In considering the results, the two sets of figures of importance in comparison are those for loss of dry matter, and the respective "nitrogen factors," at the end of 48 days, by which time the decompositions have become very slow. Series A, B and C fall together for comparison, since B consists of the flax shives removed from A in the operation of scutching after the retting process. Retting is, of course, a biological process by which the fibre bundles are loosened from the wood. In the first stage the soluble materials are fermented away by aerobic organisms, producing thereby anaerobic conditions in the tanks and paving the way for the main anaerobic fermentation of

Table III. *"Nitrogen factor" of various materials at several stages of decomposition.*

Series No.	Plant material	Time in days	Total N %	NH_3-N %	Organic N %	Loss of dry matter %	Organic N per 100 g. original	"Nitrogen factor"	Nitrogen equivalent
A	Flax straw (normal)	—	0·51	—	0·51	—	—	—	—
		8	1·46	0·15	1·31	19·54	1·05	0·54	2·76
		16	1·50	0·07	1·43	24·22	1·08	0·57	2·35
		24	1·68	0·17	1·51	31·10	1·04	0·53	1·70
		48	1·91	0·22	1·69	42·62	0·97	0·46	1·08
B	Flax shives (retted)	—	0·21	—	0·21	—	—	—	—
		8	0·54	0·19	0·35	5·14	0·33	0·12	2·33
		16	0·53	0·19	0·34	12·90	0·30	0·09	0·70
		24	0·70	0·31	0·39	16·76	0·32	0·11	0·66
		48	0·77	0·36	0·41	19·63	0·33	0·12	0·61
C	Flax fibre	—	0·55	—	0·55	—	—	—	—
		8	0·88	0·19	0·69	1·96	0·68	0·13	6·63
		16	1·28	0·29	0·99	9·16	0·89	0·34	3·71
		24	1·22	0·21	1·01	18·98	0·82	0·27	1·42
		48	1·48	0·27	1·21	28·29	0·87	0·32	1·13
D	Willow peelings	—	1·58	—	1·58	—	—	—	—
		8	2·67	0·32	2·37	2·77	2·30	0·72	26·0
		16	3·01	0·52	2·49	6·54	2·32	0·74	11·3
		24	3·05	0·32	2·73	14·20	2·34	0·76	5·3
		48	3·34	0·26	3·08	15·39	2·61	1·03	6·7
D2*	Willow peelings (without added nitrogen)	8	1·82	Nil	1·82	6·20	1·70	0·12	(1·93)
		16	2·01	Nil	2·01	12·22	1·76	0·18	(1·47)
		24	2·22	0·06	2·16	15·33	1·83	0·25	(1·63)
		48	2·21	0·05	2·16	16·93	1·79	0·21	(1·24)
E	Oat straw	—	0·30	—	0·30	—	—	—	—
		8	1·91	0·71	1·20	12·58	1·05	0·75	5·96
		16	1·56	0·40	1·16	24·90	0·97	0·67	2·69
		24	1·92	0·25	1·67	37·03	1·05	0·75	2·03
		48	2·54	0·36	2·18	50·44	1·10	0·80	1·38
F	Oat straw extracted with hot water	—	0·27	—	0·27	—	—	—	—
		8	1·65	0·86	0·79	2·05	0·77	0·50	24·4
		16	1·61	0·81	0·80	10·02	0·72	0·45	4·41
		24	1·53	0·77	0·76	13·82	0·66	0·39	2·82
		48	1·71	0·74	0·97	17·79	0·80	0·51	2·86
G	Oat straw extracted with 4 % NaOH	—	0·12	—	0·12	—	—	—	—
		8	1·70	1·51	0·19	8·86	0·17	0·05	0·45
		16	1·49	1·27	0·22	14·53	0·19	0·07	0·48
		24	1·18	0·82	0·36	16·84	0·30	0·18	1·07
		48	0·88	0·53	1·35	21·32	0·28	0·16	0·74
H	Oat straw extracted with 2 % HCl	—	0·34	—	0·34	—	—	—	—
		8	1·64	1·24	0·40	4·24	0·38	0·04	0·94
		16	1·67	1·29	0·38	6·76	0·35	0·01	0·14
		24	1·43	0·76	0·67	8·32	0·61	0·27	3·24
		48	1·11	0·44	0·67	9·14	0·61	0·27	2·95
I	Oat straw extracted with 4 % alc. NaOH	—	0·11	—	0·11	—	—	—	—
		8	1·26	0·88	0·38	11·24	0·34	0·23	2·05
		16	1·28	0·94	0·34	16·72	0·28	0·17	1·02
		24	1·47	0·97	0·50	19·26	0·40	0·29	1·31
		48	0·98	0·39	0·59	21·11	0·47	0·36	1·70
J	Barley straw extracted with 4 % NaOH	—	Nil	—	Nil	—	—	—	—
		8	1·22	0·95	0·27	15·16	0·23	0·23	1·52
		16	1·08	0·60	0·48	17·67	0·39	0·39	2·20
		24	1·20	0·56	0·64	18·81	0·52	0·52	2·76
		48	0·76	0·21	0·55	19·66	0·44	0·44	2·24
K	Esparto grass waste	—	0·95	—	0·95	—	—	—	—
		28	2·03	0·14	1·89	21·93	1·48	0·53	2·41
		56	2·05	0·07	1·98	25·65	1·47	0·52	2·03
L	Esparto grass waste extracted with 4 % alc. NaOH	—	0·23	—	0·23	—	—	—	—
		28	1·13	0·41	0·72	23·20	0·55	0·32	1·38

* Nitrogen absorbed as ammonia from incubator.

pectin which results in the separation of the bundles. In the flax shives, therefore, there must be an absence of soluble and easily fermentable material, and a close agreement in the contents of the main structural constituents, since these are unaffected in the retting process. The figures obtained on the rotted materials show a very marked difference, considerably larger than anticipated in view of the resemblance in composition. The straw suffered a loss of over 40 % and the shives less than 20 %, the nitrogen immobilised by the latter being only one-fourth of that locked up by the former. This would suggest that the absence of readily soluble and fermentable material tends to lower the "nitrogen factor." The flax fibre, which is completely deficient in soluble materials and consists mainly of cellulose, suffers a 30 % loss, at the same time immobilising 0·32 % of nitrogen. The fibre being non-lignified is in theory wholly available, but nevertheless decomposition is not extensive nor nitrogen immobilisation large. Possibly this is due to the fact that the range of active organisms is rather restricted since the medium is almost completely cellulosic.

The same general trend will be seen in the oat straw series, E–I. The loss of organic matter in the case of water-extracted straw is unaccountably low. The hemicellulose content of this material is high, and from the analytical figures a decomposition considerably more extensive would have been expected. In spite of this, however, the "nitrogen factor" is fairly high, being 0·5 as opposed to 0·8 in the case of normal straw. In Series G the straw was extracted extensively with 4 % NaOH, and this is seen to have the effect of removing the major part of the hemicelluloses, as evidenced by the low pentose and uronic acid figures, while in addition the lignin content is considerably lowered. The composition of the resulting material approaches that of the flax fibre, the differences being that it still contains pentose material intimately associated with the cellulose and is partially lignified. The absence of soluble materials and the presence of lignin together limit the extent of decomposition to a little over 20 % and the "nitrogen factor" to the low figure of 0·16. This may also be seen in Series H, in which all soluble and hydrolysable material was removed by boiling for a considerable period with 2 % HCl, a process which does not remove lignin to any extent. The final composition of this extracted material resembles that of certain woods. The total loss of organic matter did not reach 10 % but the "nitrogen factor" was a little higher than expected, being 0·27. In Series I, the oat straw was extracted with 4 % NaOH in 55 % alcohol, a treatment designed to remove much of the lignin without extracting the hemicelluloses. The lignin content was lowered from 18·5 to 8·2 %, but a portion of the hemicelluloses was also removed. In later work, the NaOH concentration was lowered to 2 % and the alcohol concentration increased to 60 %. The decomposition after this treatment was rather more than 20 % and the "nitrogen factor" 0·36. It was noticed that this treatment resulted in an unusual physical condition of the straw, and, however prolonged the washing with alcohol and cold water, the straw remained very gelatinous when wet.

Series J was a young barley straw cut 10 weeks after the appearance of the first tiller and exhaustively extracted with 4 % NaOH, a procedure which resulted in the production of a material giving analytical results very similar to those of the flax fibre. The figures on decomposition also resembled those of flax fibre, there being a 20 % decomposition and a "nitrogen factor" of 0·4.

To examine again the effect of extraction with alcoholic NaOH and the result of lowering the lignin content by this means, esparto grass waste was treated in this way. It will be seen in K and L that the loss of organic matter in the treated and untreated is about equal, but that the "nitrogen factor" is lowered in the former case from 0·5 to 0·3. That decomposition is not more extensive is no doubt due to the physical condition of the extracted material.

All the above mentioned materials contained an insufficient supply of nitrogen as evinced by their positive "nitrogen factors"; in the absence of added nitrogen their decomposition would have been slow or in some cases negligible in extent. The willow peelings, however, must be considered separately. In Table I a "nitrogen factor" of 1·33—the highest ever observed—was recorded for a sample of this material. This particular sample contained originally 1·9 % N, an amount so large that it would be expected to be more than sufficient for complete decomposition. In fact, it might be expected that losses of nitrogen would occur during the rotting process. Nevertheless, in the presence of added inorganic nitrogen an additional amount of 1·33 % per 100 g. was retained. A second sample, distinctly more woody in nature, was obtained, the analytical figures for which are given under D in Table II. It will be seen that the lignin estimation by the 72 % H_2SO_4 method gave 36·5 %. On decomposition in the presence of added ammonia, just over 15 % of organic matter was lost and 1·0 g. nitrogen immobilised per 100 g. In the absence of added nitrogen rather more organic matter was lost. There was, however, a slight pick-up of nitrogen from the ammonia lost by the other bottles in the incubator. This, however, does not obscure the salient point, which is that this material, though already containing ample nitrogen for decomposition will cause the immobilisation of a considerable additional amount without, however, undergoing a more extensive decomposition. In fact, the presence of ammonia seems even to have retarded decomposition in the early stages. It is clear that the protein or nitrogenous material of the willow peelings, although available, is less so than the ammonia, which is utilised preferentially.

Similar observations have been made in the case of certain other substances given in Table IV.

The retarding effect of an unnecessary addition of nitrogen in the form of ammonium carbonate is seen in the case of the maize straw decompositions, while actual losses, shown by a negative "nitrogen factor" occur in the case of the bean husks which have a very high initial nitrogen content.

The change in the "nitrogen factor" as the decomposition proceeds is of

Table IV. *"Nitrogen factors" of certain materials already containing sufficient nitrogen for decomposition.*

Plant material		Original nitrogen content %	Organic nitrogen content of rotted material %	Loss of organic matter %	"Nitrogen factor"
Rice straw (abnormal manurial treatment)	No N	0·31	0·59	25·36	0·13*
	With N	0·31	1·37	25·37	0·63
Maize straw (Brazil)	No N	1·02	1·61	36·7	—
	0·5 % N	1·02	1·79	31·53	0·20
	1·0 % N	1·02	1·86	28·31	0·31
	2·0 % N	1·02	2·21	26·20	0·28
Bean husks:					
Sample I	With N	3·00	6·48	44·08	0·62
Sample II: (*a*)	No N	2·99	4·80	48·31	−0·51
(*b*)	No N	2·99	4·64	47·16	−0·54
(*a*)	With N	2·99	5·40	42·54	0·11
(*b*)	With N	2·99	5·38	40·93	0·18

* Absorbed from ammonia in incubator.

some interest, since it emphasises the fact that the nitrogenous figures represent an equilibrium between immobilisation on the one hand and ammonification on the other. The equilibrium point may change during the course of a decomposition, for as the various plant constituents are removed and by-products formed, there is a sequence of active forms. Under the conditions of the experiments given above, relative stability would have been reached before the end of 48 days. A higher "nitrogen factor" may be recorded earlier as shown in Series A (flax straw) or in other cases there may be a fall followed by a rise.

II. *"Nitrogen equivalent."*

The "nitrogen factor" is an expression which contains no direct reference to the efficiency of the organism or organisms in decomposing the plant material in question, or inversely, to the availability of the material. Two plant materials may be found to have the same "nitrogen factor," say 0·5, but one may have lost in decomposition 50 % and the other only 20 %. Obviously the organisms in the former case were more efficient per unit of nitrogen, the plant material being readily available. If, however, the nitrogen requirements are re-calculated on a basis of equal amounts of organic material fermented away, a figure is obtained which measures the efficiency of the microbial tissue in decomposition. The term "nitrogen equivalent" is suggested for this factor, some of the theoretical aspects of the use of which have been described in Part IV of this series. In the example given above, the former material would have a "nitrogen equivalent" of 1·0 and the latter of 2·5, indicating the difference in availability of the two materials. The "nitrogen equivalent" may be defined as the nitrogen immobilised in the course of the removal of 100 g. of organic matter from any material. When determined at any given time of decomposition, this factor also is a summation of the

microbial activities up to that point. If the decomposition is effected by a mixed general flora, ammonifying organisms by liberating ammonia from dead microbial tissue undoubtedly reduce the total immobilised nitrogen and render both the "nitrogen equivalent" and the "nitrogen factor" apparent rather than absolute. If rapid ammonification takes place it is possible that a portion of the nitrogen may be active, that is, present as living protein in active microbial tissue more than once during the decomposition. This, however, does not wholly invalidate the use of the "nitrogen equivalent" for mixed flora decompositions, though it must be admitted that its chief value is for pure culture decompositions. As a standard for comparison the figures for Series E, oat straw, may be taken, the initial "nitrogen equivalent" being about 6, falling after 48 days to 1·4. The following figures for rice straw were calculated from those given by Rege [1927] and show the same trend in more detail.

Table V. *"Nitrogen equivalent" of rice straw in decomposition (Rege).*

Time in days	4	8	12	16	20	24	28	36	40
Loss of dry matter (%)	4·4	8·8	24·6	30·2	36·1	39·2	41·4	43·2	45·0
"Nitrogen factor"	0·12	0·53	0·88	0·90	0·92	1·02	0·93	0·91	0·90
"Nitrogen equivalent"	2·71	6·01	3·58	2·98	2·55	2·60	2·24	2·10	2·00

The standard "nitrogen equivalent" likely to be reached by mixed flora decomposition of a common straw is about 1·5, it being assumed that it has a "nitrogen factor" of 0·75 and suffers a loss of organic matter of 50 %.

The effect of extraction of the several plant materials on the "nitrogen equivalent" does not appear to be so regular as in the case of the "nitrogen factor." The removal of easily soluble materials in the case of the straw extracted with hot water caused a high initial "nitrogen equivalent" falling at the close to about 3, a figure distinctly higher than that for the normal straw. In the case of the flax shives the initial "nitrogen equivalent" is only a little over 2, falling ultimately to 0·6. There is similarly a considerable difference in trend between the highly cellulosic materials in Series C, G and J, indicating differences in availability which cannot be assessed by any method of chemical analysis.

Conclusions.

To determine the relationship, if any, between the chemical composition of a material and the quantity of inorganic nitrogen temporarily immobilised during decomposition was the purpose of these experiments, and in this only partial success has been achieved. It is possible to indicate approximately what the "nitrogen factor" is likely to be, knowing the general composition of the material, but any absolute prediction is not possible for at least two reasons. In the first place the mechanical and physical differences in structure cannot be determined or taken into account, but must affect considerably the biological availability of the tissue. In the second place, the nitrogen picture is obscured by the fact that it is only possible to determine the changes

in the quantity of added inorganic nitrogen immobilised. It is impossible to learn whether the natural proteins of the plant tissue are also utilised. The evidence in the case of Series D, willow peelings, would suggest that in the presence of a sufficiency of inorganic nitrogen, they are not utilised, but there is no means of being certain on this point. It is possible that plant proteins are as readily ammonified as microbial tissue and that in the presence of ammonifiers, the normal condition in the case of a mixed flora, the ammonia liberated would be utilised by other organisms. If this is the case, the actual total "active" nitrogen would be considerably in excess of the "nitrogen factor" and the latter would merely be the difference between the amount of nitrogen liberated from the plant proteins and the total nitrogen requirement of that plant material in decomposition. If that is so, then the amount and availability of the nitrogenous substances of the plant is of great significance in determining the "nitrogen factor." As "availability" is impossible to ascertain, any prediction on these grounds is of doubtful value.

It seems that a high "nitrogen factor" is likely to be given by a substance containing about 50 % Cross and Bevan cellulose, not more than 20 % lignin, a fairly high content of hemicelluloses as measured by free pentose content, and a low nitrogen content, provided that it also contains water-soluble material. The removal of the water-soluble material seems markedly to affect the "nitrogen factor," and plant wastes that have been submitted to extraction with water or removal of these substances by biological means, although otherwise suitable, are likely to have a "nitrogen factor" considerably lower. A highly cellulosic material low in nitrogen will have a "nitrogen factor" of 0·3 to 0·4, while high lignification depresses the factor, since decomposition is impeded.

Summary.

1. No direct relationship was found between the composition of plant materials and the amount of additional available nitrogen immobilised during decomposition ("nitrogen factor").

2. The nitrogen factor represents only the equilibrium between immobilisation on the one hand and ammonification on the other, and does not necessarily represent the whole of the nitrogen active in decomposition, since the plant proteins may also be attacked and utilised in part, or microbial nitrogen may be liberated and re-utilised.

3. Plant materials already containing sufficient or more than sufficient nitrogen for decomposition may nevertheless immobilise an additional amount, owing to preferential utilisation of the inorganic form.

4. If the nitrogen requirements of materials in decomposition are related on the basis of equal amounts of organic matter removed, a figure is obtained which expresses the efficiency of the organism or mixed flora effecting the decomposition. This "nitrogen equivalent," like the nitrogen factor is a summation of the microbial activities and is apparent rather than absolute.

Certain of the results quoted here were obtained by Mr R. L. Amoore, to whom our thanks are due.

One of the authors (A.G.N.), is indebted to the Department of Scientific and Industrial Research for a Senior Research Award.

REFERENCES.

Hutchinson and Richards (1921). *J. Min. Agric.* **28**, 398.
Norman (1929). *Biochem. J.* **23**, 1353.
—— (1931). *Ann. App. Biol.* **18**, 244.
Rege (1927). *Ann. App. Biol.* **14**, 1. (Rothamsted Memoirs, **12**, No. 43.)
Richards and Hutchinson (1924). *Eng. Pat.* 219, 384.

CXCII. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

VI. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE RATE OF IMMOBILISATION OF NITROGEN BY STRAW.

By ARTHUR GEOFFREY NORMAN.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

(*Received September 9th, 1931.*)

The total nitrogen requirements of any plant material in decomposition may be determined quite simply, and can conveniently be expressed by some such term as the "nitrogen factor." This term was first employed by Hutchinson and Richards [1921], and may be defined as the inorganic nitrogen temporarily immobilised by 100 g. of material in decomposition. Certain considerations which affect the nitrogen factor have been discussed in the preceding paper of this series [Richards and Norman, 1931]. The rate of immobilisation has, however, not been determined, nor has the influence of hydrogen ion concentration upon the rate been investigated. Any differences either in rate or extent of decomposition which may be observed will, of course, be due to a modification of the active flora. It might be expected, therefore, that both the rate of fermentation and the amounts of the various constituents removed might vary considerably with changes in the p_H of the medium.

It was necessary in the first place to devise some means by which the hydrogen ion concentration of the decomposing material could be kept approximately constant, and by which a sufficiency of available nitrogen would be present at all stages of the decomposition, without having a large excess in the initial stage since this might interfere with the normal course of decomposition. Both these requirements pointed to some arrangement for the continuous supply of nitrogen.

Richards showed that a solution of ammonium carbonate (10 parts of nitrogen per 100,000), could be passed through a percolating filter of wheat straw until, on the twentieth day, only 1 % of the ammonia nitrogen would be found in the effluent and all but a small percentage (3·4) of the balance retained in the filter as microbial protein. From the twentieth day the mature filter would continue to remove practically all the nitrogen fed to it until saturation point was reached, *i.e.* until the "nitrogen factor," 0·7 per 100 parts of dry straw, had been attained. This laboratory experiment and the subse-

quent successful trials on the large scale made by Richards and Weekes [1921] with sewage were carried out at a p_H of 7·0–8·0.

Accordingly straw was arranged in the form of a filter and supplied with a very dilute solution of an ammonium salt adjusted to the required p_H. This appeared to give very satisfactory results, and is a method suitable for other studies of a similar nature.

Experimental.

Known amounts of dry chaffed straw were packed into a series of lamp glasses as supplied for paraffin lamps, the wider end being covered by muslin held in place by rubber bands. The most convenient size of lamp glass held about 50 g. straw, but others rather larger holding 70 g. were also employed and were quite satisfactory.

A dilute solution of an ammonium salt together with certain mineral salts, adjusted to the required hydrogen ion concentration, was allowed to drop through at the rate of 1 litre per day. At the concentration of nitrogen employed (7 parts per 100,000) this was sufficiently in excess for all stages of the decomposition. Even at the most active point less than 50 % of the nitrogen was retained while in general 10 to 15 % only was immobilised. The mineral salts were added in case the leaching effect of a continuous flow removed any essential ions. The potash, for example, is easily washed out of straw[1]. The rate of flow maintained was sufficient to keep the p_H close to the desired point, and quite insufficient to cause the development of anaerobic conditions. The distribution was found to be very satisfactory after the first two or three days. By means of the Nessler reaction the amount of ammonia in the effluent was checked daily. This was merely a precautionary measure to ensure the presence of sufficient nitrogen. The results obtained were not sufficiently accurate to be regarded as a measure of immobilisation, since they do not show the amount biologically immobilised by conversion to microbial protein, but merely the amount retained or lost by the filter.

At appropriate intervals, as indicated roughly by the Nessler figures, a whole filter was taken down and the following determinations made:

1. Loss of dry matter.
2. Total nitrogen content—Kjeldahl.
3. Ammoniacal nitrogen content—distillation with MgO.

After drying and grinding, the following determinations were made:

4. Total furfuraldehyde yield.
5. Cellulose—Cross and Bevan.
6. Furfuraldehyde from Cross and Bevan cellulose.
7. Carbon dioxide yield, uronic acids.

[1] Over 90 % of the total K_2O in wheat straw is soluble in water, and in barley straw 97 %. (Richards and Rege, unpublished.)

From the first three the nitrogen factor and nitrogen equivalent of the straw at any point could be calculated and compared with the losses sustained by the various constituents.

Four series of experiments were carried out, the variations of ingoing nitrogenous solution being indicated below. It was not found possible to hold the p_H of the solutions more closely to the desired figure than the limits given below, since it was undesirable to introduce buffering salts.

Series I. p_H = 8·5–8·8, N as ammonium carbonate.
,, II. p_H = 7·4–7·8, N as ammonium chloride + NaOH.
,, III. p_H = 5·0–5·5, N as ammonium chloride + 2 cc. acetic acid per litre.
,, IV. p_H = 7·4–7·8, N as ammonium chloride + sucrose to give 26 parts carbon per 100,000 + NaOH.

In each case the necessary amount of ammonium salt to give 7 parts per 100,000 on dilution was dissolved in 1 litre of distilled water together with the following mineral salts:

K_2HPO_4	...	...	2·0 g.
$MgSO_4$	...	...	1·0 g.
NaCl ...	...	...	0·2 g.
$FeCl_2$ ...	...	...	0·05 g.

The p_H was adjusted so that when 10 cc. of the above solution were diluted to 1 litre the required p_H was given. This was a matter of some difficulty, since the distilled water was usually slightly acid and showed some variation, thereby accounting for the variations recorded above. The p_H of the effluent was determined from time to time. These figures will not be given in detail for their absolute value is doubtful. The following are the extreme limits of variation recorded. Usually the figure was nearer that of the ingoing solution. The alkaline filters tended to become more nearly neutral, the neutral filters slightly acid, and the acid filters less acid, though never reaching neutrality.

Series I. Effluent p_H = 7·8.
,, II. ,, p_H = 6·8.
,, III. ,, p_H = 6·6.
,, IV. ,, p_H = 6·4.

When thoroughly wet each filter was inoculated with 100 cc. inoculum from soil containing decomposing organic matter.

The results of analyses are given in the following Tables. Table I shows the nitrogenous changes at various periods for the four series; Table II contains the analytical figures calculated on the basis of 100 g. of original straw.

Table I. *Nitrogen content of straw filters.*

Day	Total N	Ammoniacal N	Organic N	Loss of dry matter	Organic N on 100 g. original straw	"Nitrogen factor"	Nitrogen equivalent
			Series I.				
—	0·302	—	0·30	—	—	—	—
6	0·47	0·03	0·44	3·19	0·43	0·13	4·07
12	0·70	0·07	0·63	3·88	0·60	0·30	7·73
16	0·80	0·10	0·70	5·10	0·66	0·36	7·06
19	0·85	0·09	0·76	11·00	0·68	0·38	3·45
23	0·87	0·12	0·75	19·77	0·60	0·30	1·52
28	0·75	0·12	0·63	21·04	0·50	0·20	0·95
34	0·82	0·13	0·69	22·69	0·53	0·23	1·01
38	0·83	0·11	0·72	24·56	0·54	0·24	0·98
42	0·98	0·11	0·87	27·91	0·63	0·30	1·07
72	1·90	0·16	1·74	48·08	0·90	0·60	1·25
			Series II.				
—	0·302	—	0·30	—	—	—	—
10	0·59	0·04	0·55	10·38	0·49	0·19	1·82
17	0·77	0·03	0·74	16·57	0·62	0·32	1·93
23	0·90	0·03	0·87	19·15	0·70	0·40	2·06
29	1·07	0·02	1·05	27·37	0·76	0·46	1·68
35	1·15	0·10	1·05	28·10	0·76	0·46	1·64
51	1·28	0·11	1·17	31·81	0·81	0·51	1·60
72	1·38	0·08	1·30	40·67	0·77	0·47	1·16
			Series III.				
—	0·302	—	0·30	—	—	—	—
10	0·57	0·03	0·54	9·74	0·49	0·19	1·99
19	0·69	0·05	0·64	15·22	0·54	0·24	1·57
23	0·74	0·04	0·70	16·98	0·58	0·28	1·65
29	0·80	0·05	0·75	20·91	0·59	0·29	1·38
37	0·90	0·04	0·86	23·48	0·66	0·36	1·53
51	0·85	0·08	0·77	30·84	0·53	0·23	0·74
72	1·15	0·07	1·08	37·48	0·68	0·38	1·01
			Series IV.				
—	0·302	—	0·30	—	—	—	—
10	0·540	0·04	0·50	4·37	0·48	0·18	4·12
17	0·80	0·05	0·75	7·15	0·70	0·40	5·60
23	0·96	0·07	0·89	14·69	0·76	0·46	3·17

Discussion of results.

(*a*) *Nitrogen immobilisation.* Differences in the rate and extent of immobilisation of nitrogen in each series may most readily be seen by comparing the "nitrogen factor" at each stage, since by this is expressed the inorganic nitrogen transformed to organic on the basis of 100 g. of original straw. Comparing first Series II and III, the neutral and acid filters respectively, it is seen that over the first 10 or 12 days very similar amounts are retained, after which period rather more is immobilised under neutral conditions. The neutral filter attains a nitrogen factor of 0·51 after 51 days falling off slightly to 0·47 at the end of 72 days. The acid filter shows a peak of 0·36 in 35 days and after a slight loss finally reaches 0·38 at the end of 72 days. Neutral conditions, therefore, result in the retention of rather more nitrogen than acid. Under slightly alkaline conditions immobilisation is more rapid during the first fortnight than in either acid or neutral conditions. A peak of 0·38

Table II. *Analyses of straw filters.*

(Expressed on 100 g. original dry straw.)

Day	Loss of dry matter	Total furfurald. yield	C. and B. cellulose	Furfurald. yield from C. and B. cellulose	CO_2 yield	Total uronic acid anhydride	Furfurald. from uronic acid anhydride	Furfurald. from pentose in hemicellulose	Xylan associated with C. and B. cellulose	"Pure" cellulose by difference
					Series I.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
16	5·10	14·15	48·23	6·45	1·21	4·84	0·80	6·90	10·0	38·2
19	11·00	12·69	43·61	6·01	0·86	3·44	0·58	6·10	9·3	34·3
23	19·77	11·90	40·01	4·89	0·84	3·36	0·56	6·45	7·6	32·4
34	22·69	11·15	37·62	4·54	0·85	3·40	0·57	6·04	7·0	30·6
42	27·91	10·19	36·61	4·49	0·85	3·40	0·57	5·13	6·9	29·7
72	48·08	6·04	23·15	2·72	0·60	2·40	0·40	2·92	4·2	18·9
					Series II.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	10·38	13·91	47·87	6·84	0·95	3·80	0·63	6·44	10·6	37·3
17	16·57	11·91	43·88	6·77	0·98	3·92	0·65	4·49	10·5	33·4
23	19·45	11·08	40·20	5·87	0·99	3·96	0·66	4·55	9·1	31·1
29	27·37	10·10	36·70	5·43	0·93	3·72	0·62	4·05	8·4	28·3
35	28·10	9·99	36·15	4·85	0·86	3·44	0·58	4·56	7·5	28·6
51	31·81	9·09	33·83	4·54	0·77	3·08	0·51	4·04	7·0	26·8
72	40·67	7·18	24·84	3·34	0·76	3·04	0·50	3·34	5·2	19·6
					Series III.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	9·74	14·13	48·91	6·80	0·99	3·96	0·66	6·67	10·5	38·4
19	15·22	13·14	43·45	6·55	0·96	3·84	0·64	5·95	10·2	33·3
23	16·98	12·62	42·40	6·33	0·93	3·72	0·62	5·67	9·8	32·6
29	20·91	11·45	38·81	5·37	0·89	3·56	0·59	5·49	8·3	30·5
37	23·48	11·27	37·61	5·02	0·96	3·84	0·64	5·61	7·8	29·8
51	30·84	10·07	33·74	4·54	0·87	3·48	0·58	4·95	7·0	26·7
72	37·48	7·86	27·54	3·79	0·78	3·12	0·52	3·55	5·9	21·6
					Series IV.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	4·37	14·05	49·64	7·09	1·17	4·68	0·78	6·08	10·99	38·7
17	7·15	13·49	47·77	6·32	1·08	4·32	0·72	6·45	9·80	38·0
23	14·69	12·26	44·26	5·72	0·95	3·80	0·63	5·91	8·87	35·4

is reached after 19 days and is followed by small losses. Finally over 72 days as much as 0·6 g. nitrogen is immobilised per 100 g. of straw. Alkaline conditions, therefore, seem ultimately even more favourable than neutral or acid, though in the intermediate period, from 4 to 5 weeks, less may be held than in the latter.

(*b*) *Loss of dry matter.* Under the conditions of the experiments the total loss of dry matter observed is not solely due to biological assimilation. In part it is due to the leaching out of soluble materials of the straw, and in part due to the removal of soluble intermediate products of microbial activity. A filter run solely on water for 51 days showed a total loss of dry matter of 21·26 %. This included biological action for no attempt was made to keep the filter sterile. There was in fact, a small "nitrogen factor" of 0·09 due to the picking up of ammonia from the air.

In the acid and neutral series losses of dry matter are very similar over the first fortnight, after which the neutral filter goes slightly ahead, and during the whole period loses rather over 40 % as opposed to 37 % in the case of the acid filter. Particularly noticeable is the regularity in the rate of decomposition.

In the alkaline filter in the early stages the rate of decomposition is slow, even though considerable immobilisation of nitrogen is taking place, and after 16 days only 5 % of dry matter has been lost. During the following weeks decomposition is very rapid, after which the rate becomes normal again. Over 72 days, 48 % is lost. The most notable feature is the well-established initial lag in decomposition, in spite of which the total final loss is greater than in either the neutral or acid series. Series IV was a short period run with a neutral sugar-nitrogen solution, in order to see how the addition of readily available carbohydrate affects the rate of decomposition of straw and the amount of nitrogen immobilised in the early stages. Even under these conditions with an excess of very readily available carbohydrate material, the loss of dry matter from the straw is as great as in the alkaline run over the same period, though the "nitrogen factor" is a little higher. Acid-production from the sugar is evident from the p_H figures. The addition of the sugar did not result in the immobilisation of as much nitrogen as might have been expected, and the experiment was discontinued.

(*c*) *Nitrogen equivalent.* The differences between the several series are well brought out by the nitrogen equivalent figures. The lower this figure, the less nitrogen has been immobilised in removing 100 g. of carbohydrate material from the straw. Both the neutral and the acid filters have similar nitrogen equivalents, that of the acid being just a little lower. The initial lag in decomposition in the alkaline filter is due to the unsuitability for cellulose decomposition of the flora developing in the opening stages, shown by the fact that the nitrogen equivalent is much higher then, being well over 7 as opposed to a maximum figure of 2 in either of the other series.

(*d*) *Loss of various constituents.* A comparison of the rate of loss of certain structural constituents shows no unusual features. The final loss of cellulose is rather greater in the alkaline filter than in either of the others as would be expected from the losses of organic matter observed. There is, however, an initial lag in the alkaline filter and even at the end of 6 weeks less has been removed than in the others. The loss of hemicellulose as measured by the disappearance of pentose presents precisely similar features, but is very different from that found to occur in straw compost heaps. Under such circumstances a very rapid fermentation of the hemicelluloses occurs in the early stages, before cellulose decomposition is appreciable. Later while the cellulose suffers a heavy loss, the hemicelluloses appear to remain at much the same level. In the filter it is true that decomposition of pentose precedes that of cellulose, but in no case does it exhibit an early peak, and losses continue at a fairly steady rate side by side with cellulose degradation

throughout the period of the experiments. The changes in the neutral filter approach most closely to those observed in compost heaps, in that the early loss of hemicelluloses is considerable but this loss is neither so large nor so rapid as would be found in the latter. The loss of xylan associated with the cellulose seems to follow closely the loss of the cellulose itself, as would be expected from its distribution.

Conclusions.

From the results presented it seems that changes in hydrogen ion concentration exercise a considerable effect on the rate and amount of nitrogen immobilised by straw undergoing decomposition. It is especially to be noted, however, that the total amount of organic matter lost may not be very different, nor the relative losses of individual constituents much affected by such changes. The wide range of organisms present in the soil and the co-operative nature of their activities make possible a large number of balanced reactions, each capable of bringing about the same ultimate result, though the route and rate of achieving it may differ. The effects and differences observed are undoubtedly due to a modification in nature of the active microflora. In the case of the acid filter, the decomposition of the straw was mainly the work of fungi, the growth of which would be favoured by such conditions; indeed a heavy growth of fungal tissue, mainly *Trichoderma*, was clearly visible in the earlier stages. There is probably a sequence of active forms as the straw undergoes modification in decomposition. The nitrogen factor in this series showed a peak of 0·36 after 5 weeks, followed by a slight fall and rise again to 0·38. The fall observed is direct evidence for the ammonification that proceeds side by side with the immobilisation of nitrogen. Ammonification may be due either to bacteria or fungi, but probably the latter. In the neutral filter the conditions are more suitable for bacterial development and not unfavourable to fungi. It is therefore not surprising that the nitrogen factor is consistently higher than under acid conditions since, in general, bacteria have a higher nitrogen content than the fungi. It is not unlikely that the fungi carry out the major part of the cellulose decomposition and that the bacteria are active in removing degradation products and synthesised fungal tissue.

Under slightly alkaline conditions it appears that the development of the fungi is hindered, and that, since the fungi are the more active in cellulose degradation, there is, in consequence, an initial lag in decomposition. It is possible that it is necessary for the conditions in the filter to become more nearly neutral before the fungi develop and that this is effected by the action of bacteria producing acid from the soluble constituents. The sudden rapid loss of organic matter which occurs at the end of a fortnight is accompanied by a considerable fall in nitrogen factor and this can be explained by fungal ammonification of the bacterial film built up in the early stages. The nitrogen

equivalent figures at this period are very significant, since they reveal the fact that the flora present suddenly becomes much more active in decomposition, implying a change of type of organism. Manure and compost heaps undergoing natural aerobic decompositions generally remain fairly alkaline or neutral, so that the maximum immobilisation of nitrogen probably occurs. The practice of adding calcium carbonate to ensure this has clearly much to recommend it, particularly in the case of composts to which ammonium sulphate has been added. However, it sometimes happens that through waterlogging or too close packing, anaerobic or partially anaerobic conditions occur, in which circumstances organic acids are produced from cellulose and the heap becomes definitely acid. Nitrogen may then be lost by ammonification and washing out of ammonium salts or by evaporation in the air. When a source of nitrogen is added in composting, as in the Adco process for the production of artificial farmyard manure, the aim is to immobilise as much nitrogen as possible in a biologically available organic condition. To achieve this it is desirable, therefore, to keep the reaction of the heap on the alkaline side. This was mentioned by Richards and Hutchinson [1924] in their patent, and the biological reasons for it are now made clear in this paper.

It is proposed to extend this work to conditions which occur in abnormal soils of various types, such as alkaline soils, very acid soils, and soils containing considerable quantities of salts.

Summary.

1. By the use of percolating straw filters supplied with available nitrogen at high dilutions, the rate of immobilisation of nitrogen during decomposition at various hydrogen ion concentrations has been determined.

2. Under slightly alkaline conditions immobilisation is more rapid in the early stages than in either slightly acid or neutral conditions, and ultimately more nitrogen is retained. Neutral conditions seem rather more favourable than acid for immobilisation.

3. In 10 weeks more organic matter is fermented away under alkaline than neutral conditions, and more under neutral than acid conditions. The alkaline filter shows an initial lag in decomposition not given by either of the others. Nitrogen equivalent figures show this to be due to the development of a primary flora relatively inactive as far as decomposition of cellulose is concerned.

4. In each case the loss of hemicelluloses is rather different from that found to occur in compost heaps, it being considerably more gradual in the filters.

5. The effects observed are due to differences in character of the active flora.

The author is indebted to Sir John Russell, Director of the Rothamsted Experimental Station for placing at his disposal the facilities of that Station,

and to the Department of Scientific and Industrial Research for a Senior Research Award, during the tenure of which this work was carried out.

Especially are his thanks due to Mr E. H. Richards, Head of the Fermentation Department, for his ready assistance and advice.

REFERENCES.

Hutchinson and Richards (1921). *J. Min. Agric.* **28**, 398.
Richards and Hutchinson (1924). *Eng. Pat.* 219, 384.
—— and Norman (1931). *Biochem. J.* **25**, 1769.
—— and Weekes (1921). *Proceedings of Engineering Conference. Institute of Civil Engineers. Section VI.*

CXCIII. EXPERIMENTS ON NUTRITION.
XI. COMPARATIVE VITAMIN B_1 VALUES OF FOODSTUFFS. FRUITS AND VEGETABLES.

By ROBERT HENRY ADERS PLIMMER, WILLIAM HENRY RAYMOND and JOHN LOWNDES.

From the Chemical Department, St Thomas's Hospital Medical School, London.

(*Received July 20th, 1931.*)

The vitamin B or B_1 values of fruits were first investigated by Osborne and Mendel [1919] who found that 5 g. of fresh apples or pears averted a decline in weight of rats, and that 10 cc. of orange juice daily gave good growth. Later [1920], they showed that 10 to 15 cc. daily of orange, lemon or grape fruit juice led to normal growth of rats. The juice of the grape was not good; some growth resulted with 2 to 5 g. of prunes. Apples and pears were then again tested and found to be a poor source of vitamin B. The poor value of grape juice has also been recorded by Randoin [1930]. The banana was shown by Sugiura and Benedict [1918] to be deficient in vitamin B. Miller [1926] found that 15 to 20 g. daily of papaya as source of vitamin B gave fair growth in rats. Experiments with orange juice have also been made by Stammers [1924] and by Willimott [1928], who confirm the earlier results that 10 cc. gave good growth but scarcely equal to the normal. The rind of lemon and grape fruit contained vitamin B as shown by Willimott and Wokes [1926]. House, Nelson and Haber [1929] stated that 3 g. of fresh tomato daily sufficed for maintenance of rats. The only tests on pigeons appear to be those by Chick and Hume [1917] who found that extracts of currants and dates had only a very slight curative effect on pigeons suffering from polyneuritis.

A fairly large number of vegetables have been examined for their comparative values in vitamin B. In all these experiments the dried vegetable was used and growth of rats was the criterion of their value. In the earlier tests the vegetable was incorporated in the food, but later a small quantity of the dried vegetable was given daily, in many experiments by Osborne and Mendel after a decline in growth of the rats had started. The results are summarised in Sherman and Smith's book [1931] and, in order to save space, are not quoted in detail here.

The older standard test, cure or prevention of polyneuritis in pigeons, for the presence of vitamin B_1 so much advocated by Chick [see Chick and Copping, 1930] has not been used since the tests on cabbage and potato by Chick and Hume [1917]. The curative method was then used: cabbage was

found to contain vitamin B, but potato was almost deficient. The preventive method has not hitherto been tried with fruits and vegetables. We have used this method throughout our series of experiments and are now able to report upon a series of about 30 common fruits and vegetables. Some of these tests were made several years ago, but to complete the series and publish as a whole was considered preferable to publishing one or more of the results separately. A clearer picture is thus revealed and reference to the matter is much easier.

Experimental.

The tests on the vitamin B_1 values of fruits and vegetables have been made on pairs of pigeons under the same housing conditions as in our previous experiments with pulses, nuts and cereals [Plimmer, Raymond and Lowndes, 1929, 1931].

It was recognised that the high water content of fruits and vegetables would occasion some difficulty in our method of testing, *i.e.* mixing the foodstuff with white rice or white flour and fishmeal. In a dried form only apple rings and apricots were obtainable; cabbage and other leaves could be dried by exposure to warm air, and roots, like carrots, could be cut up into small cubes. Our trials with these foodstuffs thus prepared and mixed with white rice and fishmeal led to no result, as the pigeons ate the rice and left the fruit or vegetable and suffered from polyneuritis. This experience led to the conclusion that these foods contained very little vitamin B_1, for we had always noticed that pigeons preferred foods containing vitamin B_1 to white rice and generally ate such foods first and left the white rice. Only boiled potato, passed through a squeezer, was readily eaten by the birds. It thus seemed that pigeons preferred fruits and vegetables in the cooked state. Cooked vegetables and fruits were therefore used as the means of procuring consumption. At the same time water could be removed by evaporation. Our procedure was to pass the fruit or vegetable through a mincer, collect all the water and solid matter and heat in a basin on a water-bath until the material became of such a consistency that it could be mixed with flour and fishmeal, and also cod-liver oil in the later tests, to form a dough which could be made into pills and dried. Pills of most of these foods were readily eaten by the pigeons; only onions seemed distasteful and acid fruits, such as rhubarb and gooseberries, were at first not palatable. This procedure was really the most suitable as it was desired to know the value of these foods in a form as close as possible to that in which they are used in human consumption. Practically all vegetables are eaten in the cooked state and fruits are eaten fresh or cooked. No objection can be made to cooking as it has been shown by many workers that vitamin B_1 is not destroyed by a temperature of 100°. All the fruits and vegetables were purchased from a local greengrocer and were the same as those sold for human consumption.

In the tests with cereals and pulses, from 20 to 40 % had to be incorporated in the diet to secure maintenance of pigeons for our test period of 26 weeks.

In the first trials with fruits and vegetables 50 % of the fresh food was mixed with the flour and fishmeal. With most the pigeons showed head retraction in periods varying from 3 to 9 weeks. It was not considered necessary to test the food in a higher proportion except when the period was about 9 weeks; 70 or 80 % was then tried. Potato was tested at 90 and 99 %. The results are shown in the following three tables under fruits; vegetables, leaves; and vegetables, roots and tubers. They are given in alphabetical order and not in the order of testing. The order of testing depended largely upon the season of the year when the material was procurable.

Table I. *Fruits.*

		Diet				Weights, g.		
Date	Time in weeks	Apple	White flour	Fish-meal	Cod-liver oil	Cock No. 98	Hen No. 62	Remarks
16. i. 29 to 13. ii. 29	4	50	47·5	2·5	0	500 375	385 270	Hen had head retraction and was cured
27. ii. 29	6					375	—	Cock had head retraction and was cured
						No. 48	No. 30	
26. iii. 30 to 30. iv. 30	5	80	18·0	1·8	0·2	410 250	295 205	Cock had head retraction and died
2. vii. 30	14					—	195	Hen died. Ate only from 3 to 10 g. daily during last 9 weeks
		Banana				No. 83	No. 78	
13. iii. 29 to 10. iv. 29	4	50	47·5	2·5	0	405 320	390 335	Cock had head retraction and died
15. v. 29	9					—	260	Hen paralysed and died
						No. 93	No. 50	
29. v. 29 to 7. viii. 29	10	70	28·5	1·5	0	395 240	435 300	Both showed head retraction. Cock died, hen cured
		Dried Plantain				No. 7	No. 8	
6. i. 26 to 10. ii. 26	5	95	0	5·0	0	590 500	450 385	No more food available Loss of weight indicates insufficient vitamin B
		Currants *red*				No. 330	No. 70	
19. vii. 28 to 15. viii. 28	4	50	47·5	2·5	0	360 295	350 275	Cock had head retraction and died
29. viii. 28	6					—	240	Hen had head retraction and was cured
		black				No. 167	No. 60	
17. vii. 29 to 4. ix. 29	7	70	28·5	1·5	0	385 270	370 285	Cock had head retraction and died
11. ix. 29	8					—	265	Hen had head retraction and was cured

Table I. *Fruits (contd.)*.

Date	Time in weeks	Diet: Gooseberries	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 330	Weights, g.: Hen No. 35	Remarks
6. vi. 28						395	415	
to		50	47·5	2·5	0			
27. vi. 28	3					355	305	Hen had head retraction and died
to								
4. vii. 28	4					325	—	Cock had head retraction and was cured
		Grapes				No. 18	No. 39	
23. x. 29						470	480	
to		70	28·5	1·5	0			
27. xi. 29	5					400	390	Cock had head retraction and was cured
to								
18. xii. 29	8					—	345	Hen had head retraction and was cured
		Orange juice				No. 66	No. 803	
16. i. 29						445	420	
to		50	47·5	2·5	0			Ate very little after 10th week
15. v. 29	17					310	285	Cock had head retraction and was cured
to								
29. v. 29	19					—	205	Hen died
						No. 18	No. 39	
13. iii. 29						490	450	
to		70	28·5	1·5	0			Five eggs laid by hen
17. vii. 29	18					435	375	Lost some weight and recovered it
to								
29. viii. 29	24					460	405	
to								
2. x. 29	29					420	380	
		Orange peel				No. 69	No. 79	
16. i. 29						400	370	
to		50	47·5	2·5	0			
13. iii. 29	8					255	300	Cock paralysed and died
to								Hen not well and not eating
8. v. 29	16					—	265	Hen died
						No. 98	No. 62	
13. iii. 29						460	360	
to		70	28·5	1·5	0			Both ate very little during whole period.
25. ix. 29	28					325	265	Lost weight in first weeks
		Plums				No. 43	No. 128	
29. viii. 28						315	395	
to		50	47·5	2·5	0			
26. ix. 28	4					240	320	Cock had head retraction and died
to								
3. x. 28	5					—	325	Hen had head retraction and was cured
		Rhubarb				No. 83	No. 96	
15. v. 29						400	320	
to		50	47·5	2·5	0			
3. vii. 29	7					250	250	Cock had head retraction and died
to								
17. vii. 29	9					—	220	Hen had head retraction and was cured

Table I. *Fruits (contd.)*.

Date	Time in weeks	Diet: Strawberries	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 17	Weights, g.: Hen No. 72	Remarks
27. vi. 28 to 8. viii. 28	6	50	47·5	2·5	0	450 310	490 345	Hen had head retraction and was cured. Cock died with large heart
		Tomatoes				No. 62	No. 43	
26. x. 27 to 30. xi. 27	5	50	47·5	2·5	0	425 345	460 330	Both showed head retraction and died
						No. 33	No. 36	
30. iv. 30 to 12. xi. 30	28	80	18	1·6	0·4	390 465	285 300	Hen laid 15 eggs; deserted
to 17. xii. 30	5	70	26·5	2·0	0·5	425	295	Cock had head retraction and died
to 28. i. 31	11					—	235	Hen had head retraction and died

Table II. *Vegetables, leaves*, etc.

Date	Time in weeks	Diet: Fresh Runner Beans	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 23	Weights, g.: Hen No. 72	Remarks
29. viii. 28 to 17. x. 28	7	50	47·4	2·6	0	385 260	455 360	Hen had head retraction and died
to 24. x. 28	8					235	—	Cock had head retraction and was cured
		Cabbage				No. 81	No. 92	
12. xii. 28 to 20. iii. 29	14	50	47·4	2·6	0	380 295	410 385	Both birds had head retraction and were cured
						No. 645	No. 50	
25. ix. 29 to 27. xi. 29	9	70	28·4	1·6	0	430 405	455 450	Cock had head retraction and was cured
to 25. xii. 29	13					—	345	Hen had head retraction and died
						No. 92	No. 91	
8. i. 30 to 4. vi. 30	21	80	18·5	1·5	0	445 395	390 360	Hen laid 10 eggs; one hatched, squab died, others deserted
to 16. vii. 30	27					410	360	Both well, but slight loss of weight
		Cauli-flower				No. 92	No. 32	
8. xi. 27 to 22. xi. 27	2	50	47·4	1·6	0	380 335	370 345	Hen died
to 13. xii. 27	5					240	—	Cock died

Table II. *Vegetables, leaves*, etc. (*contd.*).

Date	Time in weeks	Diet: Celery	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 17	Weights, g.: Hen No. 16	Remarks
19. x. 27						445	430	
to		50	47·5	2·5	0			
16. xi. 27	4					305	285	Hen died
to								
23. xi. 27	5					325	—	Cock had head retraction and died
		Kale				No. 74	No. 75	
12. xii. 28						390	365	
to		50	47·4	2·6	0			
6. ii. 29	8					310	255	Hen was paralysed and died. Cock had head retraction and died
						No. 645	No. 63	
1. v. 29						415	345	
to		70	28·4	1·6	0			
17. vii. 29	11					335	245	Hen had head retraction and was cured
to								
8. viii. 29	14					335	—	Cock had head retraction and was cured
		Lettuce				No. 89	No. 49	
11. vii. 28						410	350	
to		50	47·6	1·4	0			
8. viii. 28	4					325	285	Both had head retraction and were cured
						No. 65	No. 58	
21. v. 30						400	340	
to		80	18·0	1·6	0·4			Two eggs hatched, squabs
16. vii. 30	8					350	360	died
to								
17. ix. 30	17					265	300	Both had head retraction and were cured
		Spinach				No. 24	No. 70	
10. x. 28						380	390	
to		50	47·4	2·6	0			
28. xi. 28	7					330	365	Both birds had head retraction and were cured
						No. 67	No. 50	
7. v. 30						375	320	
to		80	18·0	1·7	0·3			
13. viii. 30	14					230	225	Cock died
to								
20. viii. 30	15					—	215	Hen died
		Sprouts (Brussels)				No. 68	No. 29	
16. xi. 27						455	350	
to		50	47·5	2·5	0			
7. xii. 27	3					415	230	Hen died
to								
14. xii. 27	4					400	—	Cock had head retraction and died
		Water-cress				No. 17	No. 45	
4. vi. 30						495	315	
to		80	18·0	1·7	0·3			Cock lost weight and recovered. Hen laid 4 eggs; deserted
13. viii. 30	10					395	330	
to								
10. xii. 30	27					455	385	
to		70	27·5	2·0	0·5			
18. iii. 31	14					290	280	Cock had head retraction and was cured
to								
20. v. 31	23					—	325	Hen died with large heart

Table III. *Vegetables, roots and tubers.*

Date	Time in weeks	Diet: Artichokes	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 38	Weights, g.: Hen No. 74	Remarks
23. xi. 27						450	390	
to		50	47·5	2·5	0			
11. i. 28	7					325	245	Cock died. Hen had head retraction and died
						No. 330	No. 35	
11. i. 28						430	440	
to		70	28·5	1·5	0			
28. iii. 28	11					410	400	Both looked ill
to								
2. v. 28	16					320	325	Cock had head retraction and was cured. Hen paralysed and was cured
						No. 61	No. 62	
28. xi. 28						450	425	
to		80	19	1	0			
13. iii. 29	15					325	405	Cock had head retraction and died
to								
5. vi. 29	27					—	325	Hen for last weeks ate from 2 to 16 g. only each day
		Beetroot				No. 26/28	No. 26/30	
14. xi. 28						460	520	
to		50	47·5	2·5	0			
19. xii. 28	5					385	325	Cock had head retraction and was cured
to								
16. i. 29	9					—	245	Hen had head retraction and died
		Carrots				No. 26	No. 78	
2. i. 29						475	455	
to		50	47·5	2·5	0			
30. i. 29	4					320	220	Cock had head retraction and was cured. Hen had head retraction and died
		Leeks				No. 38	No. 44	
31. x. 28						345	435	
to		50	47·5	3·5	0			
27. ii. 29	17					275	525	Cock died
to						No. 72		
20. iii. 29	20					400	470	New cock
to								
24. iv. 29	25					290	380	Hen had head retraction and died
to								
5. v. 29	27					245	—	Cock was paralysed and died
						No. 337	No. 38	
16. x. 29						415	305	
to		60	37·5	2·5	0			
18. xii. 29	9					420	305	Cock escaped
to								
15. i. 30	13					—	245	Hen had head retraction and was cured
						No. 84	No. 2	
8. i. 30						590	310	
to		60	37·5	2·5	0			
19. ii. 30	7					415	230	Cock had head retraction and died. Hen was paralysed and died

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Leeks	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 19	Weights, g.: Hen No. 20	Remarks
19. ii. 30						450	375	
to		80	18·5	1·5	0			
19. iii. 30	4					490	385	
to		80	18·0	1·6	0·4			Leeks more fibrous
2. vii. 30	19					350	275	Hen had head retraction
to								and was cured
9. vii. 30	20					310	—	Cock had head retraction and was cured
		Onions				No. 38	No. 82	
8. v. 29						375	360	
to		50	47·5	2·5	0			Birds ill in 5th week
17. vii. 29	10					245	255	Hen had head retraction and died. Cock was paralysed and died
		Parsnips				No. 331	No. 28	
21. xii. 27						455	470	
to		50	47·5	2·5	0			
7. iii. 28	11					330	335	Hen was paralysed and
to								died
6. vi. 28	24					245	—	Cock had head retraction and died
						No. 24	No. 70	
2. i. 29						410	435	
to		70	28·4	1·6	0			
6. iii. 29	9					410	450	
to		50	47·5	2·5	0			
27. iii. 29	3					320	345	Hen had head retraction and was cured. Cock was very ill
						No. 81	No. 13	
1. v. 29						380	320	
to		50	47·5	2·5	0			
17. vii. 29	11					245	295	Cock had head retraction
to								and was cured
18. ix. 29	20					—	225	Hen died
						No. 56	No. 14	
31. x. 28						430	430	
to		60	38·0	2·0	0			Started with 2 squabs which died. Hen laid 6 eggs
15. v. 29	28					350	395	Cock had head retraction
to								and was cured
29. v. 29	30					375	365	Both well
						No. 92	No. 91	
1. v. 29						420	385	
to		60	38·0	2·0	0			Two squabs hatched but showed head retraction in 13th week
18. ix. 29	20					315	240	Hen laid 3 eggs. Loss of
to								weight from poor dry parsnips. Recovered on
13. xi. 29	28					410	355	fresh parsnips

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Potatoes	Diet: White rice	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 24/17	Weights, g.: Hen No. 24/16	Remarks
10. ix. 24						385	370	
to		97·3	0	2·7	0			
31. xii. 24	16					385	415	Both well
to		82·5	16·5	1·0	0			
25. iii. 25	12					410	470	
to		79·2	19·8	1·0	0			Four eggs laid by hen
17. vi. 25	12					320	285	
to		98·8	0	1·2	0			
5. viii. 25	7					395	240	Hen died
to								
9. ix. 25	12					240	—	Cock died
						No. 25/8	No. 25/12	
23. ix. 25						385	380	
to		99·0	0	1·0	0			
14. x. 25	3					460	430	
to		82·4	16·5	1·1	0			
3. iii. 26	20					445	440	
to								
12. v. 26	30					395	205	Hen died
to								
14. vii. 26	39					230	—	Cock died
						No. 5	No. 4	
16. vi. 26						405	425	
to		99·0	0	1·0	0			Four eggs during first
3. xi. 26	20					505	445	5 weeks
to								
23. ii. 27	36					480	430	
			White flour			No. 23	No. 50	
5. xii. 28						425	480	
to		70·0	28·5	1·5	0			
30. i. 29	8					340	400	Both had head retraction. Cock died; hen was cured
						No. 37	No. 338	
16. x. 29						420	390	
to		80	17·5	2·5	0			
8. i. 30	12					315	310	Cock had head retraction and died
to								
9. iv. 30	25					—	315	Hen had head retraction and died
						No. 339		
19. ii. 30						365	—	
to								
7. v. 30	11					315	—	Had head retraction and died
							No. 98	
9. iv. 30						—	335	
to								
11. vi. 30	9					—	235	Had head retraction and was cured
						No. 31	No. 32	
11. xii. 29						395	400	
to		90	8·6	1·4	0			
7. v. 30	21					395	405	
to								
4. vi. 30	25					255	370	Cock had head retraction and was cured
to								
25. vi. 30	28					—	310	Hen had head retraction and was cured

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Swedes	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 88	Hen No. 407	Remarks
18. i. 28 to		50	47·5	2·5	0	515	405	
22. ii. 28 to	5					295	250	Cock had head retraction and died
7. iii. 28	7					—	235	Hen died
		Turnips				No. 26	No. 8	
24. x. 28 to		50	47·5	2·5	0	440	325	
21. xi. 28 to	4					315	240	Cock had head retraction and was cured
12. xii. 28	7					—	205	Hen died

The birds were cured by giving a dose of 1 g. of wheat germ, or of marmite.

Discussion.

Fruits. In no case could the pigeons be maintained with the fruit composing 50 % of the diet. With 70 % of the diet composed of fruit maintenance of the pigeons was not longer than from 9 to 11 weeks, except in the cases of orange juice and orange peel. With 80 % of tomatoes the pigeons were maintained for our test period of 26 or more weeks.

The maintenance with orange peel was remarkable as the birds lost weight during the first few weeks and kept at this level for the rest of the test period. The birds ate very little during the whole time. Though the test indicates that orange peel is equal to orange juice in vitamin B_1 value, the general appearance of the birds leads to doubt. We would prefer to suggest that the oil in the rind had some medicinal action. In our experience with autopsies of pigeons which have died from too little vitamin B the intestines have contained dark food mixed often with bile. There have usually been signs of decomposing food in the intestines and it is possible that the essential oil of the orange peel has prevented decomposition and the birds have not been poisoned by a toxin. Such poisoning is a possible cause of the symptom of head retraction. Vitamin B_1 acts by preventing the formation of the toxin from stagnant food in the gut. Evans and Lepkovsky [1929] have noted a sparing action of fat on the requirement of vitamin B_1, and Plimmer, Rosedale and Raymond [1927] found that the fat of the diet required balancing by vitamin B. The fat may have acted partly as lubricant preventing stagnation of the food in the gut. Of the various roots which we have tested parsnips have shown the highest value. These vegetables contain a peculiar essential oil. The vitamin B_1 values of fruits are very small.

Vegetables, leaves. There does not appear to be any similarity in the values of the green leaves. Pigeons were maintained on the diet with 80 % of cabbage and 80 % of watercress. Lettuce and spinach at 80 % and 70 % failed to secure maintenance for 26 weeks. Brussels sprouts were not tested

above 50 %, as this amount indicated very little vitamin B_1. Fresh runner beans probably contain the vitamin B_1 in the seed and not in the green pod, which forms the bulk of the edible matter of fresh beans.

In general, vegetable leaves have a better vitamin B_1 value than vegetable roots and fruits.

Vegetables, roots and tubers. Of the roots and tubers tested, artichokes, leeks, parsnips and potatoes contain some vitamin B_1. Beetroot, carrots, swedes and turnips contain very little. Parsnips at the level of 60 % maintained the pigeons, but the value may be due partly to the essential oil.

The experiments with potatoes were rather variable. At first it seemed that the pigeons could be maintained on 80 % with white rice, but on re-testing with white flour 90 % was scarcely sufficient. The potatoes were tested in different years, and it is very likely that vegetables vary slightly according to the season. The water content of vegetables is higher in wet seasons [Plimmer, 1921] and accordingly there may be a variation in vitamin B_1 value. Young and old samples, as observed with parsnips, would also give a variation in vitamin B value. Another variation may arise from the soil in which the vegetables are grown, and whether the soil has been manured or not manured [*cf.* McCarrison, 1926; Rowlands and Wilkinson, 1930], as well as the kind employed.

Regarding the value of vegetables and fruits as a source of vitamin B_1 in the general human dietary, our results do not indicate that they have any important value. In order to supply sufficient vitamin B_1 from 60 to 80 % of the diet must be composed of these foodstuffs in the fresh state, amounts which are impossible for man to consume. On the average the consumption of fruits and vegetables is from 4 to 8 oz.; only potatoes may form a considerable part of the human daily diet (from 1 to 2 lb.). The requirement of man for vitamin B_1 is probably not the same as that of the pigeon, and even if it is the same as that of the rat as estimated by Plimmer, Rosedale and Raymond [1927] the amount of vegetable usually eaten by man is not alone enough for his vitamin B_1 requirement.

Summary.

In comparison with cereals and pulses, fresh fruits and vegetables have a much lower vitamin B_1 value, but if allowance be made for their high water content, the comparison is more favourable. Table IV (p. 1799) shows the water content, percentages of wet and dry material in the diets, maintenance figure (+ or −) and comparative vitamin B_1 values with dried yeast, our standard, cereals and pulses.

It is difficult to compare these figures with those of the other workers with rats. On the whole they correspond fairly closely in relation to dried yeast, being from 1/5th to 1/10th the value of this material [see Roscoe's comparison, 1930].

Table IV.

	Water content %	Percentage amount in diet		Maintenance	Comparative vitamin B_1 value	
		Wet	Dry		Wet	Dry
Dried yeast	0	.	4	+		100
Cereals	.	.	40	+		10
Pulses	.	.	30	+		13
Fruits:						
Apple	85·3	50	13·0	−		
		80	37·0	−		
Banana	70·8	50	22·5	−		
		70	40·0	−		
Currants, red	85·7	50	12·6	−		
,, black	80·8	70	31·0	−		
Gooseberries	89·3	50	10·0	−		
Grapes	84·7	70	26·0	−		
Orange juice	87·0	50	11·5	−		
		70	23·0	+	6	20
,, peel	78·9	50	18·0	−		
		70	33·0	+	6	13
Plums	85·7	50	13·0	−		
Rhubarb	94·4	50	5·0	−		
Strawberries	90·1	50	9·0	−		
Tomatoes	93·5	50	6·0	−		
		70	14·5	−		
		80	20·7	+	5	20
Vegetables, leaves:						
Beans, runner	93·2	50	12·0	−		
Cabbage	88·0	50	11·0	−		
		70	22·0	−		
		80	32·4	+	5	13
Cauliflower	90·2	50	16·4	−		
Celery	93·7	50	11·0	−		
Kale	83·3	50	14·4	−		
		70	28·0	−		
Lettuce	95·4	50	4·4	−		
		80	15·6	−		
Spinach	92·8	50	6·7	−		
		80	22·5	−		
Sprouts	88·3	50	11·0	−		
Watercress	93·9	70	12·5	−		
		80	20·0	+	5	20
Vegetables, roots etc.*:*						
Artichokes	79·7	50	17·0	−		
		70	32·0	−		
		80	45·0	+	5	9
Beetroot	90·2	50	17·0	−		
Carrots	87·0	50	11·5	−		
Leeks	85·3	50	12·9	−		
		60	18·0	−		
		80	37·0	+	5	10
Onions	94·2	50	5·5	−		
Parsnips	72·6	50	21·3	−		
		60	35·0	+	7	12
Potatoes	80·5	70	31·0	−		
		80	44·0	−		
		90	64·0	+	4	7
Swedes	88·8	50	10·0	−		
Turnips	93·0	50	6·5	−		

We wish to express our grateful acknowledgment for grants from the Government Grant Committee of the Royal Society and from the Imperial Chemical Industries Limited, which have defrayed the expenses of these experiments.

REFERENCES.

Chick and Copping (1930). *Biochem. J.* **24**, 1764.
—— and Hume (1917). *J. Roy. Army Med. Corps*, **29**, 121.
Evans and Lepkovsky (1929). *J. Biol. Chem.* **83**, 269.
House, Nelson and Haber (1929). *J. Biol. Chem.* **81**, 495.
McCarrison (1926). *Indian J. Med. Res.* **14**, 351.
Miller (1926). *Biochem. J.* **20**, 515.
Osborne and Mendel (1919). *Proc. Soc. Exp. Biol. Med.* **17**, 46.
—— —— (1920). *J. Biol. Chem.* **42**, 465.
Plimmer (1921). Analyses and energy values of foods, p. 157 (H.M. Stationery Office).
—— Raymond and Lowndes (1929). *Biochem. J.* **23**, 546.
—— —— —— (1931). *Biochem. J.* **25**, 691.
—— Rosedale and Raymond (1927). *Biochem. J.* **21**, 913.
Randoin (1930). *Brit. Chem. Abstr.* 1931, A, 269.
Roscoe (1930). *Biochem. J.* **24**, 1754.
Rowlands and Wilkinson (1930). *Biochem. J.* **24**, 1754.
Sherman and Smith (1931). The vitamins, 2nd ed. (Chemical Catalog Co., New York).
Stammers (1924). *Biochem. J.* **18**, 9.
Sugiura and Benedict (1918). *J. Biol. Chem.* **36**, 171.
Willimott (1928). *Biochem. J.* **22**, 67, 535.
—— and Wokes (1926). *Biochem. J.* **20**, 1299.

CXCIV. THE SIGNIFICANCE OF THE MICHAELIS CONSTANT FOR SACCHARASE.

By GEORGE EDWARD BRIGGS.

From the Botany School, Cambridge.

(*Received August 20th, 1931.*)

Michaelis and Menten [1913] suggested that the relation between the rate of hydrolysis of sucrose by saccharase and the substrate concentration could be best interpreted if it was assumed that one molecule of enzyme combined with one molecule of substrate to form a complex, which broke down into free enzyme and fructose and glucose. Adopting this schema Briggs and Haldane [1925] showed that the rate of hydrolysis (A) should be related to the concentration of enzyme (E) and the concentration of substrate (S) by the following expression

$$A = \frac{k_3 E.S}{S + (k_2 + k_3)/k_1},$$

where k_1 is the velocity constant of combination of free enzyme and substrate, k_2 of the reversion of the complex to free enzyme and substrate, and k_3 of the breakdown of the complex into free enzyme and products. The fundamental difference between this expression and that suggested by Michaelis and Menten is that, here, the Michaelis constant, K_m, is $(k_2 + k_3)/k_1$, whilst Michaelis and Menten used k_2/k_1, the dissociation constant of the enzyme-substrate complex. In other words, the assumption that k_3 is negligible compared with k_2 is implicit in the expression of Michaelis and Menten, which is now widely adopted. This question of the relative dimensions of these constants is of considerable importance. Calculations of the heat of dissociation of the complex from data on the relation between K_m and temperature [Euler and Laurin, 1920; Dann, 1931] have significance only in as far as k_3 is negligible.

The constant k_3 is itself quite large. Haldane [1930] suggests that it is about 2000 for saccharase at 15°—that is, one molecule of saccharase is capable of hydrolysing 2000 molecules of sucrose per second. Our own estimates from data of Euler and his co-workers suggest a figure about 100.

Knowledge of the relative dimensions of k_2 and k_3 might be gained from a study of the change of rate of hydrolysis starting from the time when the enzyme and substrate were mixed to the time when the maximum rate was reached. As far as we are aware no such study has, as yet, been made and we shall not consider the theoretical aspects of this. What we do propose to

consider is the light thrown on this question by the data on the relation between the rate of hydrolysis and substrate concentration at different concentrations of hydrogen ions.

Michaelis and Davidsohn [1911] found that the relation between rate of hydrolysis of sucrose by saccharase and p_H was almost identical with the relation between the undissociated fraction of a weak acid and p_H. On the basis of this they suggested that saccharase, on the alkaline side of p_H 4·6, behaved as a weak acid and that only the undissociated part combined with sucrose to form a complex capable of forming fructose and glucose. With regard to this picture Haldane says, "It is clear that Michaelis' theory is incomplete, though it has been, and still is, a valuable approximation to the facts." Subsequently Michaelis and Rothstein [1920] found that the rate of hydrolysis changed with p_H in the same manner whatever the concentration of sucrose, in other words K_m was independent of p_H. This caused them to modify the earlier picture to the view that saccharase "verbindet sich mit Saccharose zu einer Verbindung, welche eine Säure ist. Die undissozierten Moleküle dieser Säure zerfallen spontan in monomolekularer Reaktion." As Kuhn [1923] rightly points out the theory does not fit the facts, and he falls back on the explanation that "Die Wasserstoffionen bestimmen die Zerfallsgeschwindigkeit des Saccharase-saccharosekomplexes." Haldane appears to be of the same opinion as Kuhn, for he states that k_3 varies twentyfold with p_H between 4 and 8[1]. As K_m does not alter with p_H he concludes "Unless then we make the quite improbable assumption that both k_1 and k_2 vary proportionally with it, the constancy of K_m shows that k_2 is large compared with k_3." This is difficult to reconcile with Haldane's previous admission that the Michaelis' theory is "a valuable approximation to the facts."

There is another possible explanation of the facts which also throws light on the relative value of k_2 and k_3. The theory embodies that of Michaelis which explains the striking similarity of the relation between rate of hydrolysis and p_H to the relation between the undissociated portion of an acid and p_H—a similarity for which Kuhn and Haldane offer no explanation when they attribute the change of rate to change of k_3 with p_H.

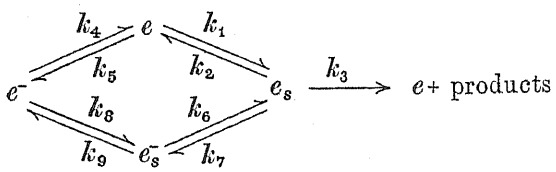
If we assume that on the alkaline side of the optimal p_H the enzyme exists in four forms (not three as the two theories of Michaelis assume)—un-ionised enzyme (e), ionised (e^-), ionised enzyme-substrate complex (e_s^-), and un-

[1] The basis for the statement about the twentyfold change of k_3 appears to be the data of Euler, Josephson and Myrbäck [1924] (quoted on p. 21 by Haldane (1930)) showing a decreased rate of hydrolysis from 100 to 5 when the p_H was increased from 5 to 8. It should be noted that Table IV in Haldane's book inverts the data, giving observed values as calculated, and *vice versa*. Further, it is the calculated values, not the observed values, which are means of the values for five different sugar concentrations at each p_H. The five values for each p_H should have been identical since they are merely the rate at that p_H relative to the value at p_H 5 calculated from the expression

$$\text{Rate} = \frac{kS}{S+k_s} \cdot \frac{H}{H+k_a}.$$

The lack of identity is due to the faulty arithmetic of Euler and his co-workers.

ionised complex (e_s)—the latter alone being capable of giving rise to products at a rate $k_3.e_s$—we can develop an expression for rate of hydrolysis in terms of the concentrations of enzyme (E), substrate (S), and hydrogen ion (H).



This is a long expression and little purpose will be served by presenting it in an unmodified form—the constants are too many for the data available. It becomes more manageable if we assume that the velocity constants of breakdown of e into e^- and H and of their re-combination are the same as the corresponding constants for e_s, the acidic dissociation constant being k_a; and a similar equality for the constants of e_s into e and S, and of e_s^- into e^- and S, the dissociation constant being k_s. So simplified, the expression becomes[1]

$$k_3 e_s = \frac{k_3 E.H}{H+k_a} \cdot \frac{S}{S+\frac{k_2+k_3}{k_1} - \frac{k_3}{k_1(H/k_a+1+S.k_1/k_5+k_2/k_5)}},$$

k_2 is the velocity constant of breakdown of e_s into e and S, and k_5 of e into e^- and H.

In the limit where H is great compared with k_a, as at the optimal p_H, we get the expression of Briggs and Haldane

$$k_3 e_s = \frac{k_3 E.S}{S+(k_2+k_3)/k_1}.$$

As k_3 becomes smaller we approach the expression

$$k_3 e_s = \frac{k_3 E.H.S}{(H+k_a).(S+k_s)}.$$

This is identical with the expression of Euler, Josephson and Myrbäck.

The form of the relation between rate of hydrolysis and substrate concentration should, then, be the same whatever the concentration of H, if k_3 is negligible—a rectangular hyperbola with ordinates proportional to $H/(H+k_a)$ and the Michaelis constant k_s or k_2/k_1 in all cases.

If k_3 is not negligible, but k_5 is small relative to k_2, we get the same expression except that the Michaelis constant is now $(k_2+k_3)/k_1$ instead of k_s or k_2/k_1. Hence the fact that the Michaelis constant is independent of p_H is, according to our schema, no evidence as to the relative sizes of k_3 and k_2, unless we know that k_5 is not small compared with k_2. The value of k_5 is not known: all we can say is that, since k_a seems to be of the order of 10^{-7}, k_4 is

[1] This expression is obtained by the solution of the four equations

$$k_1 eS + k_4 e_s^- H - e_s(k_2+k_3+k_5) = 0,$$
$$k_5 e_s + k_1 e^- S - e_s^-(k_4 H + k_2) = 0,$$
$$k_5 e + k_2 e_s^- - e^-(k_1 S + k_4 H) = 0,$$
$$e + e^- + e_s + e_s^- = E.$$

about ten million times as big as k_5. If k_2 is of the same order as k_3, about 2000 according to Haldane, then, unless k_4 is of the order of 10^{10}, k_5 will not be great compared with k_2.

It seems worth while considering the effect of p_H on the Michaelis constant assuming that k_5 is not small compared with k_2 but actually as great as that quantity, and k_3 as much as a half of k_2.

The data of Kuhn on the effect of change of p_H on the relation between rate of hydrolysis and concentration of sucrose seem as reliable as any. They seem to lend support to the view that the Michaelis constant is independent of p_H. It is on the basis of these results that Kuhn draws the conclusion about the effect of p_H on the breakdown of the enzyme-substrate complex. The question to be decided is as to whether the results can be explained on the basis of our schema with k_3 half as great as k_2, and the latter equal to k_5. Of course, as we have pointed out above, if k_5 is small compared with k_2 we should expect the Michaelis constant to be independent of p_H, no matter what the size of k_3 relative to k_2. At p_H 4·4–4·6 Kuhn found that the relation between rate of hydrolysis and substrate concentration could be fitted with a rectangular hyperbola with the Michaelis constant 0·040 M. He also concludes that the results at p_H 6·79 are fitted with the constant at the same value. Making our assumptions as to the relative values of k_2, k_3 and k_5 the relation between rate of hydrolysis (A) and substrate concentration at p_H 6·79 should be

$$A = \frac{S}{S + 0{\cdot}04 - 0{\cdot}04/3\,(3 + 37{\cdot}5\,S)},$$

taking H as equal to k_a. The results calculated on the basis of this expression and the observed values, along with those calculated by Kuhn assuming the rate proportional to $S/(S + 0{\cdot}04)$, are given in Table I.

Table I. *Rate of hydrolysis of sucrose by saccharase at* p_H *6·79 (after Kuhn).*

Concentration of sucrose S	Rate of hydrolysis				
		Calculated			
	Observed	I	II	III	IV
0·134 M	18	17	17	78	77
0·066	14	14	14	64	62
0·0415	11·5	11·5	11·5	53	51
0·0252	9	9	9	41	39
0·0128	5·5	5·5	5·5	26	24
0·0073	3·5	3·5	3·5	17	15·5

Calculated I and III from expression $\frac{S}{S + 0{\cdot}04 - 0{\cdot}04/3\,(3 + 37{\cdot}5\,S)}$.
Calculated II and IV from $S/(S + 0{\cdot}04)$.
I and II reduced to observed at 0·066 M, III and IV as percentages of rate when S is very great.

The observed rates are clearly given by Kuhn as correct to half a unit, a large quantity for the smaller rates. The rates were determined from curves of hydrolysis against time, by interpolation to find the amount hydrolysed in 10 minutes. The rates are intended as measures of the initial velocity,

before the substrate concentration has changed, and when there are no products of hydrolysis to interfere with the combination of enzyme and substrate. These progress curves are practically straight lines for 20 minutes or more with the higher concentrations of substrate, and hence there is no great difficulty in ascertaining the initial velocity, but with the smaller concentrations the rate falls away more rapidly. An inspection of the progress curves makes one realise that the change in 10 minutes under-estimates the rate of hydrolysis at the lower concentrations, and that in any case the values are certainly not reliable to within less than half a unit. In both sets of calculations the figures have been reduced, so that the calculated rate agrees with the observed when the substrate concentration is 0·066 M and the figures are given as correct to the nearest half-unit. To multiply the observed values and give them correct to 0·5 % as Kuhn does, is unjustifiable. As reduced by the first method the calculated results by both methods are identical.

Hence until we know that k_5 is considerably greater than k_2 we cannot conclude that results such as those of Kuhn, which seem as accurate as any at present, are incompatible with the constant k_3 being comparable in magnitude with k_2, in other words we cannot conclude that the Michaelis constant is equal to the dissociation constant of the enzyme-substrate complex.

There is the possibility that Haldane intends k_3 to be taken as the average rate of breakdown of the complex of enzyme and substrate no matter whether it is ionised or not. In that case, since it appears that only the un-ionised portion of the complex breaks down into products, k_3 or k_h, as we will call it to avoid confusion, is an average of a finite quantity and zero. If

$$H_s = e_s + e_s^- \text{ and } H = e + e^-,$$

the rate of hydrolysis will be given by the expression

$$A = \frac{k_h E.S}{S + (k_2 + k_h)/k_1}.$$

Since the rate of hydrolysis varies with p_H it seems that k_h must, and further since $(k_2 + k_h)/k_1$ does not, it appears that k_h must be small compared with k_2, as Haldane argues. But according to our schema $k_h = k_3.e_s/(e_s + e_s^-)$, and from equations on p. 1803 and the expression for e_s when k_5 is small compared with k_2 we find that

$$k_h = k_3 \cdot \frac{S + k_2/k_1}{\{S + (k_2 + k_3)/k_1\}(1 + k_a/H) - k_3/k_1}.$$

The constant k_h is, then, a function of S as well as of H, except when H is very large, when $k_h = k_3$. Putting this value for k_h in the expression

$$A = \frac{k_h E.S}{S + (k_2 + k_h)/k_1}$$

we get back to our expression

$$A = \frac{k_3 E.H.S}{(H + k_a)\{S + (k_2 + k_3)/k_1\}}.$$

Thus we see that arguments from an expression using the average rate of breakdown of the enzyme-substrate complex (including ionised as well as un-ionised) can be misleading as to the relative dimensions of k_3 and k_2.

We do not suggest that Haldane uses this particular argument, for we are not sure what precise significance he attaches to k_3, but we cannot admit that his conclusion that k_3 is negligible compared with k_2 is warranted by the facts he produces.

REFERENCES.

Briggs and Haldane (1925). *Biochem. J.* **19**, 338.
Dann (1931). *Biochem. J.* **25**, 177.
Euler and Laurin (1920). *Z. physiol. Chem.* **110**, 55.
—— Josephson and Myrbäck (1924). *Z. physiol. Chem.* **134**, 39.
Haldane (1930). Enzymes. (Longmans, London.)
Kuhn (1923). *Z. physiol. Chem.* **125**, 28.
Michaelis and Davidsohn (1911). *Biochem. Z.* **35**, 366.
—— and Menten (1913). *Biochem. Z.* **49**, 333.
—— and Rothstein (1920). *Biochem. Z.* **110**, 217.

CXCV. STUDIES ON ANTIGLYOXALASE.
II. METHODS OF FOLLOWING GLYOXALASE ACTION.

By JUOZAS OSJA GIRŠAVIČIUS.

From the Biochemical Laboratory, Cambridge.

(*Received August 26th, 1931.*)

Criticism of the manometric method.

In a previous paper [Giršavičius, 1930] an attempt at following glyoxalase action by a manometric method was described. It was thought that the evolution of CO_2 in a bicarbonate-CO_2 buffer system would provide a convenient means of following the production of lactic or mandelic acid under the action of glyoxalase. It was soon found, however, that a gas evolution, indistinguishable from that caused by the investigated reaction, takes place when phenylglyoxal and pancreatic extract are brought together. Further investigation showed that this reaction is not abolished by first boiling the pancreatic extract, and that boiled extracts of testis and of yeast show the same phenomenon, whilst boiled liver and kidney extracts do not. As the reaction is abolished after treating the tissue extract with nitrous acid, or after precipitation with phosphotungstic acid, it was considered to be due to amino-compounds. It was then shown that, although monoamino-acids do not cause more than a slight gas evolution with phenylglyoxal, diamino-acids cause a rapid gas evolution; the conclusion was drawn that diamino-acids or related compounds, or polypeptides containing a high proportion of diamino-acids, are responsible for the reaction in question. Later work has shown, however, that proteins and peptones also cause a gas evolution in the Barcroft manometer, and that this reaction is apparently characteristic for all protein derivatives, although with monoamino-acids it is comparatively slow.

The difference between liver and kidney extracts on the one hand and extracts of pancreas, yeast and testis on the other hand seems to be due to differences in the amount of non-heat-coagulable N they contain, rather than to the proportion of diamino-acids entering into their composition.

Exp. 1. In the following experiment the effects of liver and of pancreatin on phenyl- and on methyl-glyoxal were compared. 0·084 *M* solutions of phenyl- and methyl-glyoxal were prepared, making use of the modified Friedemann method described in this paper.

0·5 cc. of these was tipped into the following solutions:

(1) with liver extract,

(*a*) 1 cc. liver extract, 1 cc. water, 0·7 cc. sodium bicarbonate solution, and, in the small cup, 0·5 cc. phenylglyoxal.

(*b*) like (*a*), but methylglyoxal instead of phenylglyoxal;

(2) with pancreatin solution (2 % pancreatin B.D.H.),

(*a*) 1 cc. pancreatin solution instead of liver extract, otherwise as (1, *a*),

(*b*) like (*a*), but with methylglyoxal.

All solutions had a p_H of 7·6. The results are given in mm.^{3} CO_2.

Minutes	(1 *a*)	(1 *b*)	(2 *a*)	(2 *b*)
4	110	60	37	14
8	154	78	47	20
15	198	98	55	29
21	225	114	—	34

Both with liver and with pancreatin the reaction proceeds at almost exactly twice the rate with phenylglyoxal as with methylglyoxal. This seems to show that not only the reaction with pancreatin, but also the reaction with liver observed under these conditions, is largely the protein reaction, rather than the reaction due to glyoxalase.

It is clear from the foregoing results that the manometric method cannot be used for following glyoxalase action. In no case is it possible to be certain how much of the reaction observed is due to glyoxalase and how much to the proteins and protein derivatives present in the solution.

In a recent paper, Lohmann [1931] has used the manometric method for a study of the co-enzyme of glyoxalase. From his results he concludes that glyoxalase preparations inactivated by dialysis, and, to a somewhat lesser extent, fresh glyoxalase preparations, are activated by the addition of a "co-enzyme" preparation. This he obtained by treating a trichloroacetic acid extract of yeast with lead acetate and mercuric nitrate. Unfortunately he gives no control figures for the effect of this preparation itself on methylglyoxal; it is therefore impossible to judge from the results he reports whether he was dealing with a true co-enzyme, or whether the activation observed was really a summation of two reactions (as when pancreatin is added to liver extract).

Since the manometric method had thus proved itself unsuitable as a means of investigating glyoxalase action, a different method had to be sought. Two ways of attacking this problem presented themselves. One could either, as Dakin and Dudley (and in some of their work Kuhn and Heckscher [1926, 1, 2] had done, estimate polarimetrically the mandelic acid produced from phenylglyoxal; or, like Kuhn and Heckscher in the main part of their work, Ariyama [1928] and Sakuma [1930], follow, by some suitable method, the disappearance of the glyoxal. As the estimation of the acid is rather laborious, the second way was chosen. It was clear, of course, that the reaction with proteins would have to be taken into account, and might interfere considerably; but whether this

effect would be large enough to make this method as unsuitable as the manometric method, could not be decided *a priori*—especially as such a method had already been used by the above authors. The first problem was to find a satisfactory way of estimating glyoxals.

Modification of Friedemann's method for use as a micro-method.

Two methods were available for estimating glyoxals; the iodimetric method of Kuhn and Heckscher, and the colorimetric method of Ariyama[1]. Of these the former seemed less satisfactory, as it is not quite specific, but mainly because of the two-hour periods required before each titration. Ariyama's method has the disadvantage that it can only be used within narrow limits of concentration with one standard. A rapid titrimetric method seemed preferable, if it could be designed. Friedemann [1927] had shown that glyoxals are quantitatively oxidised by hydrogen peroxide in alkaline solution giving two equivalents of acid for each mol. of glyoxal. Phenylglyoxal, for instance, is oxidised to an equimolar mixture of benzoic and formic acids. Friedemann based on these facts a method for estimating various glyoxals by titrating the acids produced. This method has not been applied in actual practice, except by Ariyama, who used it to standardise his methylglyoxal solutions. It seemed probable that if it could be modified into a micro-method of sufficient accuracy and sensitivity, Friedemann's method would have the advantage of being very rapid and simple to carry out.

For accurate acidimetric titration of dilute solutions it is necessary to determine the end-point with reference to a standard. It is also necessary to control the indicator concentration, which can be done in two ways: either the standard is diluted parallel with the titrated fluid, or else more indicator is added during the titration, so as to keep its concentration constant. The first method is preferable, as coloured solutions can be titrated fairly accurately by its use. It is convenient to carry out the titrations in front of a box with a plate glass front illuminated from inside.

The following solutions are placed in five boiling-tubes: *A*, *B*, *s*, *1*, *2*.

A, CO_2-free water, containing 1 cc. phenolphthalein per 10 cc., and adjusted to a rose-pink colour with a few drops of *N*/50 NaOH.

B, 20 vol. H_2O_2 diluted 1 : 5, containing 1 cc. phenolphthalein per 10 cc., and adjusted to the same colour as *A*. The colour of this solution gradually fades on standing, owing to destruction of the indicator; from time to time a few drops of phenolphthalein have to be added, to readjust the colour.

[1] Recently Barrenscheen and Dreguss [1931] have published another method for the estimation of glyoxals. They claim it to be very specific, and, judging by their figures, it seems to be more sensitive and perhaps more accurate than the method described in this paper. On the other hand, it is far more cumbersome. Where specificity, or great accuracy at high dilution, are required, the method of Barrenscheen and Dreguss (or Ariyama's method) will be preferable. Where these considerations are less important, the method here described will probably be sufficiently accurate and at the same time simpler and more rapid.

s, *1*, *2*, receive 10 cc. each of the solution to be titrated, and 1 cc. phenolphthalein. *s*, which serves as standard for the titration, and the two other tubes, are then adjusted to the same colour as *A* and *B*.

1 cc. *A* is now added to *s*, and 1 cc. *B* to *1* and *2*. If the solution contains any methyl- or phenyl-glyoxal the colour in *1* and *2* will fade or disappear immediately on adding the hydrogen peroxide. *1* is then titrated with *N*/50 NaOH to a permanent red colour. The same amount of NaOH is added to *2* (it is convenient to have two burettes attached to the bottle containing the NaOH); to *s* is added the same volume of CO_2-free water. After standing about a minute, tubes *1* and *2* are adjusted to the colour of *s* with *N*/50 HCl, again with addition of CO_2-free water to *s*. It is clear that the sharpness of the end point will depend upon the absence of substances exerting a buffer action within the p_H range of the indicator used. With phenolphthalein as indicator phosphate has little buffering power. The buffer action of borate can be shifted into a lower p_H range by adding a glycerol solution to tubes *s*, *1*, *2*. On the other hand, carbonates present in the original solution, and CO_2 absorbed from the atmosphere, definitely diminish the sharpness of the readings. The figures of Table I may serve to illustrate the accuracy obtainable.

Table I.

Phenylglyoxal cc.	NaOH (found) cc.	NaOH (average) cc.	NaOH (calculated) cc.
3	6·08	6·06	—
3	6·02		
3	6·09		
1	2·03	2·01	2·02
1	1·99		
0·5	1·00	1·02	1·01
0·5	1·04		
0·5	1·05		
0·5	1·01		
5	2·10	2·13	—
5	2·16		
5	2·16		
5	2·13		
5	2·10		
2	0·84	0·86	0·85
2	0·87		
2	0·88		
2	0·84		
1	0·44	(0·43)	0·42
1	0·43		
1	0·42		
1	(0·61)		
1	0·42		

As 1 mol. of the various glyoxals gives rise to 2 equivalents of acid, 1 equivalent of NaOH corresponds to 36 g. methylglyoxal or 67 g. phenylglyoxal. 1 cc. *N*/50 NaOH corresponds to 0·72 mg. methylglyoxal, or 1·34 mg. phenylglyoxal.

For the estimation of phenylglyoxal in solutions containing tissue extracts, 25 cc. are pipetted into 50 cc. volumetric flasks, containing 2 cc. 10 % trichloroacetic acid. After standing for some minutes the liquids are made up

to the mark and filtered through dry pleated filters (Dreverhoff's "Rapid Filtrierpapier" is very convenient) into small dry flasks. Samples of 10 cc. are then pipetted out for analysis, and treated as described above. Separate experiments have shown that the concentration of phenylglyoxal is not altered by precipitating protein in the solution and filtering.

The main defect of the present method lies in its lack of specificity. Acetaldehyde does not interfere. Although it is gradually attacked under the conditions of this method, the reaction is so slow that even high concentrations of acetaldehyde introduce no appreciable error. On the other hand pyruvic acid and dihydroxyacetone behave exactly like methyl- and phenyl-glyoxal, and it is possible that this method may be of some use in their estimation, where confusion is out of the question. The possible confusion with dihydroxyacetone, and particularly with pyruvic acid, of course diminishes considerably the usefulness of the method for the estimation of glyoxals.

Investigation of glyoxalase action by following glyoxal disappearance.

In the work now to be described the modified Friedemann method has been made use of for following the disappearance of phenylglyoxal under the action of glyoxalase and under various other conditions. The following typical experiment on the action of pancreas will serve to illustrate the method as used in practice.

Exp. 2.

Solution (1): 20 cc. liver extract, 20 cc. phosphate buffer, $p_H = 7{\cdot}6$, 20 cc. water.
Solution (2): like solution (1), but instead of water 20 cc. pancreatic extract.

These solutions were incubated overnight at 38°[1]. 25 cc. phenylglyoxal solution were then added to each and samples were taken for analysis immediately and after 40 and 80 minutes. The following are the results, the figures being the differences between the initial and final titrations (cc. $N/50$ NaOH):

Initial phenylglyoxal = 6·49 cc. NaOH.

Minutes	(1)		(2)	
40	0·20	0·29	0·54	0·68
80	—	0·67	0·84	0·86

It will be seen that in the present experiment not only does the addition of pancreas not cause any inhibition, but in its presence actually more phenylglyoxal disappears than without it. The following experiment explains this phenomenon.

Exp. 3.

Solution (1): 50 cc. liver extract, 15 cc. buffer, 60 cc. water.
Solution (2): 50 cc. water, 15 cc. buffer, 60 cc. 0·4 % pancreatin B.D.H.
Solution (3): 50 cc. liver extract, 15 cc. buffer, 60 cc. pancreatin.

The solutions were incubated for $5\frac{1}{2}$ hours. 50 cc. of each were then pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately and after 1 hour.

[1] In this as in all following experiments toluene was added as an antiseptic.

Initial phenylglyoxal = 4·27 cc. $N/50$ NaOH.

Phenylglyoxal destroyed after 1 hour equals (in cc. $N/50$ NaOH):

(1)		(2)		(3)	
0·95	0·93	0·19	0·18	1·17	1·10

It will be seen that the destruction of phenylglyoxal by liver and by pancreatin alone have been exactly summed, when both were incubated together; there was neither inhibition nor acceleration. It is, however, possible to demonstrate antiglyoxalase action, as the following experiments with a different sample of pancreatin show.

Exp. 4.

Solution (1): 50 cc. liver, 20 cc. water, 20 cc. buffer.

Solution (2): 50 cc. water, 20 cc. pancreatin solution, 20 cc. buffer.

Solution (3): 50 cc. liver, 20 cc. pancreatin solution, 20 cc. buffer.

After incubation overnight 30 cc. of these solutions were pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately, after 35 minutes and after 1 hour and 30 minutes.

Initial phenylglyoxal = 2·58 cc. $N/50$ NaOH.

Minutes	(1)	(2)	(3)	(3)-(2)
35	0·52	0·06	0·39	0·33
150	1·50	0·33	0·76	0·43

In the fourth column the figures obtained by subtracting the phenylglyoxal destruction by pancreatin alone from the phenylglyoxal destruction by liver *plus* pancreatin, are tabulated. Whether this correction is applied or not, the figures show a considerable inhibition.

Exp. 5. In this experiment a larger amount of pancreatin was used. The solutions were treated as in the preceding experiment; the results alone will therefore be given.

Initial phenylglyoxal = 6·92 cc. $N/50$ NaOH.

The amounts of phenylglyoxal destroyed after 1 hour equal:

(1) Liver alone	(2) Pancreatin alone	(3) Liver *plus* pancreatin	
2·26	2·04	2·36	2·33

If the destruction of phenylglyoxal by pancreatin is quantitatively summed in (3) with the residual activity of the liver extract, almost complete inhibition is shown by these figures. This experiment, however, also shows clearly the reason why this method was finally abandoned as unsuitable for the study of glyoxalase action. The destruction of phenylglyoxal by pancreas alone is far too large to be ignored, in fact the correction that has to be applied for it is in many experiments considerably larger than the effect investigated—the inhibition by antiglyoxalase. It is impossible, under these conditions, to obtain reliable results.

Moreover, other experiments have shown that, as with the gas evolution measured manometrically, the destruction of phenylglyoxal by pancreatin and

pancreatic extracts is only a special case of a reaction with proteins and their derivatives. It is clear therefore that not only has the destruction of phenylglyoxal by liver treated with pancreas to be corrected for the destruction by pancreas alone, but, which is even more important, a considerable and not readily assessable proportion of the phenylglyoxal destruction by liver extracts (and those of other tissues) is due, not to glyoxalase, but to a reaction with the tissue proteins. This makes it impossible to interpret with any certainty the results obtained. It can be concluded from these experiments that it is impossible to follow glyoxalase action in more than the roughest qualitative manner by following the disappearance of the substrate. Glyoxalase can only be studied reliably by estimating the lactic or mandelic acid produced.

Much of the work so far done on glyoxalase (Kuhn and Heckscher, Ariyama, Sakuma) has depended on the estimation of glyoxals by various methods. The results obtained in this way have to be accepted with some care, as they cannot be relied upon to represent actual glyoxalase action. Only the work carried out with Dakin and Dudley's technique is free from this particular objection.

The rather irregular kinetics found by Kuhn and Heckscher [1926, 1] in their study of methylglyoxalase, and the variable results these authors obtained, for instance on dialysing their extracts, are probably partly to be explained by the phenomena described above. The p_H-activity curves obtained by Kuhn and Heckscher (fall in activity beyond p_H 8), as well as the diminishing activity of their extracts on standing, show that their results are at any rate partly, perhaps mainly, due to glyoxalase action. Non-enzymic methylglyoxal destruction must, however, have gone on simultaneously, so that their results cannot be interpreted with certainty. Kuhn and Heckscher [1926, 2] also noted the destruction of methylglyoxal by boiled tissue extracts, causing an apparent acceleration of glyoxalase, but did not follow up this observation. Sakuma's work calls for similar comments. This author observed the destruction of methylglyoxal by proteins and various amino-compounds. According to his observations the reaction is a rather complex one; simultaneously with the disappearance of methylglyoxal there is an equivalent production of NH_3 and CO_2. This probably explains the gas evolution observed when using the manometric method, which in the previous paper of this series was tentatively, and apparently erroneously, ascribed to the action of some acid compound of phenylglyoxal and an amino-acid. Whilst Sakuma has studied this reaction in some detail, he has failed to see that it greatly diminishes the value of his work on the distribution of glyoxalase, and on the "co-enzyme" of glyoxalase. That such a co-enzyme is active in boiled tissue extracts, like those of Sakuma, is possible. In view of the work of the Neuberg school it is even quite probable. His results do not, however, prove the existence of such a co-enzyme, especially as he gives no control figures for the destruction of methylglyoxal by his "co-enzyme" solutions alone. In his Table V, for instance, boiled extracts of liver, muscle, and kidney are added to a liver and to a muscle extract. The percentage activations obtained differ widely, being far greater with the weakly active muscle extract. If, however, the differences, in other words the excess

activities when boiled extracts are added, are compared instead, it is found that each of the boiled extracts produces practically the same increase in activity, whether it is added to the liver or to the muscle extract. Again, if the results of his Table VI, where increasing amounts of boiled liver juice are added to liver extracts, dialysed for various periods of time, are plotted, it will be seen that the points fall practically on parallel straight lines, differing only in their point of origin, according to the activity of the liver extracts themselves. These results are more consistent with a summation of activities, as in Exp. 3 and others of the present paper, than with a co-enzyme activation. At any rate the question is still open. The inhibition by phosphate and the exceedingly interesting acceleration by sugars, which Sakuma reports, are more likely to be properties of glyoxalase itself. But all these require re-investigation.

It will be seen from the present paper, that only results obtained by estimating the acid produced can give a true picture of glyoxalase action. So far the polarimetric estimation of mandelic acid is the only method available for this purpose. This method, as used by Dakin and Dudley, is rather cumbersome. Some improvements have therefore been introduced, and will be described in a subsequent paper, together with the results obtained.

Summary.

1. Manometric methods, based on Warburg's glycolytic technique, are unsuitable for the study of glyoxalase as glyoxals cause a gas evolution with proteins and their derivatives.

2. A volumetric method for the estimation of glyoxals is described. This method makes use of the fact, discovered by Friedemann, that glyoxals are oxidised to two equivalents of acid by H_2O_2 in alkaline solution.

3. By means of this method it is shown that, owing to its relatively high rate, the reaction between glyoxals and proteins cannot be ignored, or even adequately corrected for. This makes results obtained by following glyoxal disappearance very unreliable as expressions of glyoxalase action. In this connection some of the work of other authors, who used similar methods, is discussed critically.

4. It follows that glyoxalase action can only be reliably investigated by estimating the acid produced.

I would like to express my thanks to Sir F. G. Hopkins and to Prof. J. B. S. Haldane for their interest and kind encouragement.

REFERENCES.

Ariyama (1928). *J. Biol. Chem.* **77**, 359.
Barrenscheen and Dreguss (1931). *Biochem. Z.* **233**, 305.
Friedemann (1927). *J. Biol. Chem.* **73**, 331.
Kuhn and Heckscher (1926, 1). *Z. physiol. Chem.* **160**, 116.
—— —— (1926, 2). *Z. physiol. Chem.* **160**, 156.
Lohmann (1931). *Biochem. Z.* **237**, 477.
Sakuma (1930). *J. Biochem.* (*Japan*), **12**, 247.
Giršavičius (1930). *Biochem. J.* **24**, 446.

CXCVI. FERMENTABLE SUGAR IN NORMAL URINE.

By VICTOR JOHN HARDING and DAVID LONGLAND SELBY.[1]

From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.

(*Received August 3rd, 1931.*)

Introduction.

The problem of true sugar in blood has now received a satisfactory solution. There may be still a little doubt as to whether a few mg. may not be a true sugar other than glucose, but within that limitation the amount can be estimated readily and accurately. It is not so with the presence of true sugar in urine. The urine of all normal persons contains carbohydrate reducing material, but to what extent this consists of glucose has been a matter of controversy. The older work has already been reviewed from time to time [Benedict, Osterberg and Neuwirth, 1918; Folin and Berglund, 1922, 2; Greenwald, Gross and Samet, 1924]. As regards more recent work, there have been no fundamentally new methods of analytical approach to the problem. Observers have still to depend on a yeast fermentation process, or on the formation of osazones.

The danger of contamination by bacteria, in using yeast for the estimation of glucose has been well recognised. Excluding this error, Lund and Wolf [1926] failed to observe CO_2 formation as a result of the incubation of pure yeasts and urine, and Patterson [1926] by incubating ordinary yeast and urine in presence of toluene noted no change in reducing power. Hiller, Linder and Van Slyke [1925] by using larger amounts of yeast shortened the time required for the removal of glucose from aqueous solutions and blood. Eagle [1927] applied the method to urine, and reported the entire absence of fermentable sugar, either fasting or after ordinary meals. His figures have received a correction from Van Slyke and Hawkins [1929], but even so the amount must be under 10 mg./100 cc. urine. On the other hand Greenwald, Gross and Maguire [1927], using Eagle's technique found fermentable sugar after meals but none after glucose in some subjects, and concluded that normal urines contain reducing substances other than glucose that are removed by treatment with yeast. In a series of after-breakfast urines Van Slyke and Hawkins have noted significant amounts of fermentable sugar. Peterson and West [1929] have also observed in 50 normal urines daily excretion of fermentable reducing substances in amounts varying widely up to 400 mg.

[1] Assisted by a grant from the Banting Research Foundation, University of Toronto.

The use of phenylhydrazine has led to similar contradictory evidence, Patterson [1926], Geelmuyden [1915], Höst [1923], have all failed to prepare *d*-phenylglucosazone from normal urines, contradicting the earlier positive claims. More recently however, Hassan [1928], removing interfering substances by charcoal, prepared typical glucosazone crystals from urine 1–2 hours after an ordinary meal in 20–30 % of 700 Egyptian students. After a 12 hours fast the occurrence of glucose was reduced to 7 %. Glucosazone may be valueless as a specific indication of glucose in urine. Quite apart from the possible presence of fructose or mannose, Greenwald, Gross and Maguire [1927] claimed the isolation of *d*-phenylglucosazone from normal urine which contained only non-fermentable carbohydrate reducing material, and drew attention to the possible formation of compounds of this type from sucrose.

A very complete examination of the problem was made by Malmros [1928] who, from evidence of osazone formation combined with yeast fermentation concluded that "even though it has not been possible to produce a chemical-analytically perfectly valid proof, the investigations still indicate that glucose occurs in normal urine."

Other investigators have approached the problem from the physiological standpoint. Thus Folin and Berglund [1922, 2] cited the evidence of blood-sugar studies after the ingestion of large amounts of pure glucose, together with the non-occurrence of any rise in the carbohydrate reducing material in the urine, against the possibility of the occurrence of glucose in normal urine. The experimental work and its value was severely criticised by Benedict and Osterberg [1923], who together with Neuwirth [1918] had put forward a view involving a continuous secretion of glucose into the urine.

Both from the clinical and the laboratory side of medicine during the last decade, there has come an increasing recognition of two types of normal people who consistently show glycosuria. The first, renal glycosuriacs [Goto, 1918; Marsh, 1921], possess a sugar threshold below the normal value and both in the fasting condition and after meals show sugar in the urine. It is recognised too that the threshold for sugar can be found at any level of the blood-sugar [Goto and Kuno, 1921; Gray, 1923; John, 1923], so that individuals will occur who show absence of glucose from fasting urine, but its presence after glucose ingestion or after meals, though the blood-sugar curve may not reach the usual normal renal threshold. The second type have been called "cyclic hyperglycaemics" [Holst, 1925; Faber, 1926] or "lag type people" [MacLean, 1924; Linder, Hiller and Van Slyke, 1924]. In them the blood-sugar rises transiently above the usual value for the renal threshold after glucose ingestion, or after meals, with resulting glycosuria.

In discussing the presence of glucose in normal urine it is usually assumed that these types are not very common, and in experiments on a small series of subjects such types are excluded by direct observation. The real problem of glucose in normal urine lies in determining whether individuals showing no clinical tests for sugar, and presumably possessing glucose tolerance curves

of the "ideal" type [MacLean, 1924; Linder, Hiller and Van Slyke, 1924], actually excrete small amounts of glucose. In view of the contradictory evidence which we have summarised, four possibilities present themselves.

(1) A difference between the fasting condition and after meals.

(2) The occurrence of subjects with thresholds for sugar lower than normal but not low enough to allow the escape of sufficient sugar to be detected clinically.

(3) The occurrence of subjects responding to glucose or carbohydrate ingestion by a hyperglycaemia above the normal threshold, but not showing sufficient glycosuria to be detected clinically.

(4) The urinary sugar reported as fermentable, or as giving *d*-phenylglucosazone, is not glucose.

Experimental.

Analytical methods.

The analytical procedures for urine are as follows:

A. Preliminary dilution. Normal urine is diluted 5 or 10 times. Urines showing positive Benedict sugar tests are diluted 20, 50 or 100 times. Very dilute urines are diluted only 1:1 or 1:2. The dilutions are arranged so that the final titration lies between the values 0·3 and 1·5 cc. of 0·005 *N* thiosulphate solution. It is to be noted that the final dilution is always twice the preliminary dilution.

B. Total "sugar." 5 cc. of the diluted urine, 5 cc. of 0·04 *N* H_2SO_4 are mixed, 0·3 g. Lloyd's reagent added; the mixture shaken and centrifuged. 2 cc. of the supernatant liquid are mixed with 2 cc. of the Shaffer-Hartmann copper solution and used as described for pure glucose under the heading "Standardisation of the copper solution."

C. Non-fermentable "sugar." 1 cc. of a 25 % suspension of washed yeast is centrifuged, the fluid adherent to the centrifuge tube is removed by filter-paper, and to the cells are added 5 cc. of the diluted urine. The cells and liquid are mixed and incubated 8 mins. at 38°, the mixture being agitated two or three times. 5 cc. 0·04 *N* H_2SO_4 are added, mixed and then 0·3 g. Lloyd's reagent, added. After shaking and centrifuging 2 cc. of the supernatant liquid are used for the determination of non-fermentable "sugar" as in total sugar.

D. Blank determination. 5 cc. water are mixed with 5 cc. 0·04 *N* H_2SO_4; 0·3 g. Lloyd's reagent added, and the mixture is shaken and centrifuged. 2 cc. supernatant liquid are used, as in determinations B or C. A blank determination must be carried out with each batch of reductions.

E. Fermentable sugar. The difference B–C properly corrected for the blank represents fermentable sugar.

Recovery of added sugar from urine. Added glucose (100 mg./100 cc.) is recovered either directly or by fermentation from all human urines so far examined. Fructose, however, in concentrations greater than 25 mg./100 cc. is not recovered quantitatively from urine by yeast under our conditions. In lower concentrations the recovery is quantitative.

In presence of a mixture of phenols representing 30 mg./100 cc. phenol the recovery of glucose is similar to that from urine.

Preparation of a modified Shaffer-Hartman micro-reagent[1].

Copper sulphate	5·0 g.
Tartaric acid	7·5 ,,
Sodium carbonate anhyd.	40·0 ,,
Potassium iodate	0·7 ,,
Potassium oxalate	18·4 ,,
Water to	1000 cc.

[1] The details of this solution and its preparation were kindly supplied to us by Prof. P. A. Shaffer. The notes and criticisms represent the experience of this laboratory after 4 years' use.

The copper sulphate and tartaric acid are dissolved in about 150 cc. of water. The sodium carbonate is dissolved in about 500 cc. of water and the iodate and oxalate in about 150 cc. of water. The solutions are all cooled to 25° and the sulphate-tartaric acid solution added slowly to the carbonate solution with gentle agitation. The temperature is not allowed to rise above 27°. The iodate-oxalate solution is then added and the whole transferred quantitatively to a volumetric flask, and brought to 1000 cc.

A separate solution of 1 % KI.

It is advisable to allow the copper solution to stand 3 weeks before use. This allows a slight amount of precipitate to settle. The solution, once made, does not alter in its oxidising power towards glucose. During the first 3 weeks the oxidising power towards the non-glucose reducing substances in blood alters slightly but after this interval it remains constant. We have used solutions over a year old, without finding any alterations in the values obtained against pure glucose, though the value of the blank increases slightly.

Standardisation of the copper solution. Solutions of pure glucose of 20, 10, 5, 2·5 and 1 mg./100 cc. are prepared.

2 cc. of the copper solution and 2 cc. of the sugar solution are mixed and heated exactly 10 mins. in a rapidly boiling water-bath in a $6'' \times \frac{3}{4}''$ tube, stoppered with a loose plug of cotton wool. The tube is cooled to 30° and 2 cc. of the KI solution are added. The I_2 is liberated by 2 cc. of N H_2SO_4 with shaking and determined by titration with 0·005 N thiosulphate solution.

We have used 1·0 % starch solution in cold saturated solution of phenol red as indicator. A blank determination with water is also carried out with each batch of reductions. The blank value is subtracted from the sugar value. The difference between duplicate titrations should not be greater than 0·02 cc. of 0·005 N thiosulphate. If desired greater or less amounts of sugar solution can be used with a corresponding increase or decrease in the volume of the reagents.

Sensitivity of the reagent. In water solution the reagents will estimate 0·1 mg./100 cc. of glucose using triplicate determinations.

Stability of reduction products. The solution besides being much more sensitive to glucose than the original Shaffer-Hartman is also superior in the stability of the cuprous compounds, formed in the reduction. The values for glucose over a range of 0·2–10·0 mg. per 100 cc. are the same in an atmosphere of N_2 as under the described ordinary conditions.

Values with blood-filtrates and urines. The method can be used with blood-filtrates prepared by the Folin-Wu method, the Somogyi precipitation with $ZnSO_4$ and NaOH, and the Herbert and Bourne method with 3 % Na_2SO_4 and tungstic acid. 0·3 cc. finger blood precipitated according to Folin-Wu was used for the figures reported in this paper.

Whilst the behaviour of the solution to glucose is constant, we have found variable results when the non-sugar fermentable material of blood is included in the determination [*cf.* Harding and Van Nostrand, 1930]. The cause of this variation we now suspect to be the bicarbonate content of our copper solution. Somogyi [1926] noted the effect of variations in the carbonate-bicarbonate ratio on the glucose values of the Shaffer-Hartman solution and as a result devised a modification containing both carbonate and bicarbonate. A similar variation evidently affects the non-sugar reducing material, as may be shown by passing into the new Shaffer-Hartman solution a stream of CO_2 and determining the values for fermentable and non-fermentable sugar in a Folin-Wu filtrate. Both are lowered, the glucose value to a greater degree than the non-fermentable value.

The values for the non-fermentable sugar in urine have varied very little from one copper solution to another prepared in the usual standard way.

Values with sugars other than glucose. The solution can be used for fructose, galactose and lactose, in water solution, or added to blood-filtrates or urine. Lactates show no reducing values nor iodine absorption values to our reagents.

Use of Lloyd's reagent for removal of interfering substances in urine previous to "sugar" determination. The necessary removal of uric acid, creatinine, *etc.* from urine previously to the application of the modified Shaffer-Hartman reagent may be effected by the original method suggested by Folin and Berglund [1922, 1] of Lloyd's reagent and H_2SO_4. It seems unnecessary to use the modifications of Folin and Svedberg [1926], or Hamilton [1928] under our conditions. The use of Lloyd's reagent, however, does necessitate the determination of a new blank value, in which water previously treated with H_2SO_4 and Lloyd's reagent under the same conditions as urine, replaces water alone.

Removal of fermentable sugar from urine by yeast. We have used Fleischman's yeast washed with water according to the directions of Somogyi [1927]. The stock washed yeast was weighed out when required to make a 25 % suspension by weight with distilled water. We have not found necessary the removal of interfering substances by Lloyd's reagent before the application of the yeast recommended by Eagle [1927].

Van Slyke and Hawkins [1929] have recently stated that yeast applied to urine not only removes fermentable sugar but in addition a variable percentage of the non-fermentable material remaining after treatment with Lloyd's reagent. This results in a correction being necessary in their technique for the determination of fermentable and non-fermentable "sugar" in urine.

We have examined our own technique to see if any such absorption of non-fermentable "sugar" is noticeable. Samples of fasting normal human urine, on which the total "sugar" was first determined, were treated with 3 successive portions of yeast as described in C and the residual non-fermentable sugar determined after each yeast treatment. The results are shown in Table I. The yeast treatments show many times that the urine has gained a little in "sugar" instead of suffering a loss as would be expected were any appreciable amount of non-fermentable yeast-absorbable substance present. The changes in either direction are small, and are of the same magnitude as the thiosulphate titration error. Thus it becomes evident that under our analytical conditions no yeast correction is necessary. Whether the difference between our results and those of Van Slyke and Hawkins lies in the higher dilutions of the urine used by us, or in the use of a copper solution as the reagent for "sugar" determination rather than a ferricyanide reagent, we do not know.

A minor but by no means negligible error can be introduced by yeast in either urines or blood-filtrates by insufficient centrifuging. A very small number of yeast cells, remaining in the supernatant liquid and thus being pipetted into the modified Shaffer-Hartman reagent, causes a marked increase in reduction. In our most accurate work we found it necessary to centrifuge the supernatant liquid a second time.

Table I. *Showing effect of successive treatments with washed yeast on total "sugar" in normal fasting human urine.*

2 cc. of 1:10 diluted urine according to B or C.

Specimen No.	Total "sugar" initial	After yeast treatment 1	2	3	Loss or gain after each yeast treatment 1	2	3
		cc. 0·005 *N* thiosulphate			cc. 0·005 *N* thiosulphate		
1	1·67	1·65	1·62	1·59	−0·02	−0·03	−0·03
2	1·29	1·25	1·27	1·28	−0·04	+0·02	+0·01
3	1·06	1·08	1·06	1·07	+0·02	−0·02	+0·01
4	0·68	0·70	0·72	0·74	+0·02	+0·02	+0·02
5	0·67	0·66	0·64	0·67	−0·01	−0·02	−0·03
6	0·64	0·64	0·64	0·64	±0·00	±0·00	±0·00
7	0·35	0·37	0·35	0·38	+0·02	−0·02	+0·03
8	0·32	0·35	0·35	0·35	+0·03	±0·00	±0·00
	Total change after yeast treatments				+0·02	−0·05	+0·07

Fasting and after glucose.

The subjects represented in Table II were medical students. No food was taken after the meal of the previous evening, the night urine was discarded at 7 a.m., 200 cc. of water were taken, and the student arrived at the laboratory a little before 9 a.m. The subject rested quietly for 15 mins. The 7 to 9 a.m. urine specimen was voided, the initial blood sample taken, and 50 g. glucose in 200 cc. water were given. Finger blood was taken every 15 mins. during the first hour and at the 30 min. interval in the second hour. After 9 a.m. the subjects performed their ordinary laboratory work. An extra 200 cc. of water

was allowed if the 7–9 a.m. specimen had been scanty. The second urine specimen was collected at 11 a.m. All analyses were performed immediately. The blood-sugar curves are shown in Figs. 1 and 2. Table II shows the difference between the total sugar and the non-fermentable "sugar" in cc. 0·005 *N* thiosulphate solution for 2 cc. of urine-filtrate, and the amount of fermentable sugar in the 2-hour sample as glucose. The small amount of thiosulphate—not more than 0·03 cc.—representing the fermentable fraction in the 7–9 a.m. specimen is in the same range of error as two duplicate determinations. The average 0·01 cc. is the same both in the fasting urine and after glucose. Subjects 5 and 9

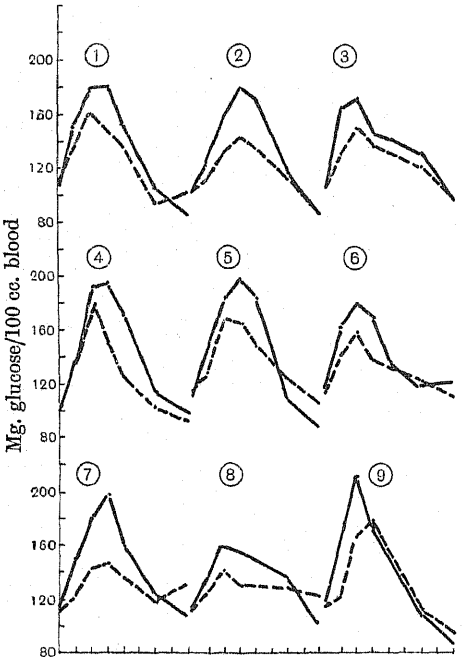


Fig. 1.

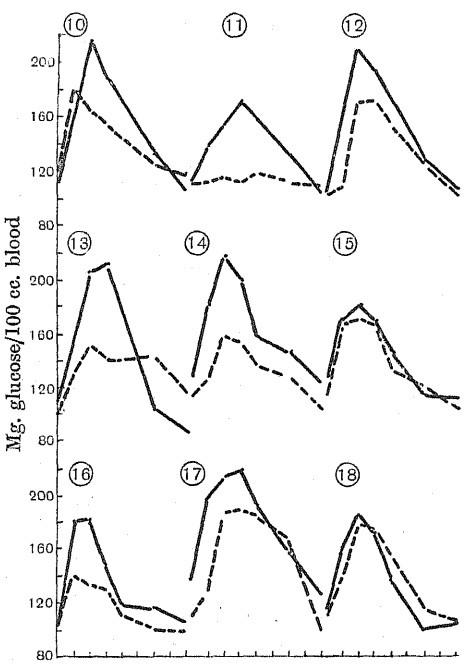


Fig. 2.

Figs. 1 and 2. Showing morning and afternoon tolerance curves after 50 g. glucose.
Morning curves ------- Afternoon curves ————

after glucose show a doubtful result. The amount is so small that the figures can at present have no significance. This does not mean to say that absolutely no glucose escapes into the urine. There may be amounts up to 2 mg./100 cc. present which is the smallest detectable by our method. Peters and Van Slyke [1931] in their recent book, summing up the available evidence, give the actual glucose content of urine as 1 mg./100 cc.

Subject 2 however, has undoubtedly allowed a few mg. of glucose to escape into the urine after glucose ingestion. His renal threshold is a little low, as the maximal blood-sugar observed after glucose ingestion was 142 mg./100 cc. (see Fig. 1). He represents however, only 1 subject out of 18

and it is evident that the occurrence of such variations is not very common. The usual "normal" possesses the usual threshold value for glucose. On the other hand relying on the osazone formation Malmros [1928] is inclined to think that 40 % represents an average rate of occurrence of physiological glycosuria. This is a percentage very much higher than ours. The difference may perhaps be racial, but may be due to our more careful exclusion of any subjects likely to be considered as mild renal glycosuriacs.

Table II.

	7–9 a.m. Fasting		9–11 a.m. 50 g. glucose at 9 a.m.	
No.	0·005 *N* thiosulphate for 2 cc. urine filtrate cc.	Fermentable sugar per 2 hr. mg.	0·005 *N* thiosulphate cc.	Fermentable sugar per 2 hr. mg.
1	−0·005	0·3	−0·01	0·5
2	+0·01	0·3	+0·09	5·1*
3	±0·00	0·0	−0·03	−3·3
4	+0·02	0·8	+0·02	2·8
5	+0·03	2·0	+0·05	2·8
6	+0·03	2·0	+0·02	1·4
7	+0·03	0·0	+0·02	1·2
8	+0·01	0·0	±0·00	0·0
9	+0·03	1·5	+0·04	3·2
10	±0·00	0·0	−0·01	0·0
11	±0·00	0·0	−0·01	0·7
12	±0·00	0·0	−0·02	1·0
13	±0·00	0·0	±0·00	0·0
14	+0·01	1·1	−0·02	−1·5
15	+0·01	0·4	−0·01	−1·8
16	±0·00	0·0	±0·00	0·0
17	−0·02	−0·5	+0·01	0·7
18	+0·01	0·0	+0·03	2·0
Average	+0·01	0·4	+0·01	0·4

* Excluded from average.

In view of the high peaks for blood-sugar shown in the morning curves in Figs. 1 and 2 by some subjects without any glucose in the urine some criticism may perhaps be directed at our analytical methods. The modified Shaffer-Hartman method of sugar analysis we employed sometimes gives slightly higher values for the non-sugar fraction of reducing material present in blood than some of the other reagents. All determinations on blood-sugar reported in this paper, however, were made with one preparation of the reagent whose value to glucose remained constant.

A further series of 15 laboratory workers has yielded us results similar to those of Table II for fasting urine and after glucose ingestion. Some of these are reported in Table I. The occasional occurrence of a few mg. of fermentable sugar in the fasting urine in the morning may perhaps represent a carry-over from the meal of the previous evening, through incomplete removal of the night urine from the bladder, rather than any physiological variation, although we have met with one subject who excreted fermentable sugar in the fasting morning urine after a late meal (midnight), when the urine flow and intake of

water seemed sufficient to ensure thorough emptying of the bladder. This same subject showed minimal fermentable sugar excretion in the 7–9 a.m. specimen if the previous evening's meal was taken 6–7 p.m. In precise work the time relation of the previous evening meal and the fasting specimen cannot be disregarded.

In the remainder of the work reported in this paper we have taken a difference of 0·05 cc. (or over) of 0·005 *N* thiosulphate, between the total "sugar" and the non-fermentable "sugar" to mean the presence of fermentable sugar. Differences less than 0·05 cc. are reported as zero. By this procedure, perhaps, we have failed to observe the excretion of some small quantities of fermentable sugar; on the other hand, we have made certain that those amounts reported as present have a real significance.

Post-prandial.

The urines represented in Table III were collected 2–4 p.m. from a similar group of students. The urines were approximately 2 hours post-prandial. Half the results were obtained in November 1929 and half in October 1930.

Table III. *Fermentable sugar in post-prandial afternoon urine.*

Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.
7	7	5	4
0	0	19	14
0	5	12	0
0	36	17	0
0	0	16	20
6	0	18	8
0	0	11	6
7	?	0	0
0	6	0	10
21	0	0	0
16	6	0	0
12	13	0	0
0	0	4	8
33	8	10	12

The results, however, are the same. About 50 % of each group show unmistakably fermentable urinary sugar. Our results thus agree in general with those of Van Slyke and Hawkins, Hassan, Peterson and West, and Malmros, that it is not an uncommon finding. The significance of the remaining 50 % who show no fermentable sugar must not be overlooked. The negative results of Folin and Berglund, Eagle, and Greenwald, Gross and Samet could obtain equal support. Post-prandial urines from 8 normal women showed 4 containing fermentable sugar.

Several questions at once arise, the most important of which is the nature of the fermentable sugar. Glucose is not the only reducing sugar removable by yeast. Fructose and mannose are also fermentable and have been shown to be partially removable by yeast under the conditions of Raymond and Blanco [1928]. Fructose in aqueous solution can be distinguished from glucose and mannose by several reactions. The most notable of these is the Seliwanoff

reaction. The diphenylamine reaction has also been used in a quantitative manner by Van Creveld [1927] and Corley [1929]. Neither of these reactions yielded satisfactory results when applied to the urines containing fermentable sugar. We consequently fell back on indirect methods of approach.

Afternoon glycosuria.

In the endeavour to find conditions under which glucose might occur in normal urine the following experiment was performed on 5 laboratory workers. Fasting urine was collected 7–9 a.m. At 9 a.m. 50 g. glucose in water or weak tea were taken followed by ordinary breakfast. Two-hour specimens were collected throughout the day until 7 p.m. Lunch was taken at 1 p.m., and at 5 p.m. 50 g. glucose were again taken. Dinner was taken at 7 p.m. with a night specimen of urine from 7 p.m. till 7 a.m. next morning. In giving glucose with an ordinary breakfast we were influenced by the claims of Benedict and his co-workers [Benedict, Osterberg and Neuwirth, 1918; Benedict and Osterberg, 1923] that glucose taken with meals behaves quite differently from glucose taken in the usual fasting condition. In view of the clear difference just exhibited between fasting and post-prandial urines, it seemed worth while to re-study the question. Greenwald, Gross and Samet [1924] had found also, both in a human subject and in a dog, that glucose given with a high protein diet produced a glycosuria. Our reason for giving 50 g. glucose in the afternoon was the accidental observation, on one of us, that glucose taken at that time produced an unmistakable amount of fermentable urinary sugar, when none was present under conditions of the usual tolerance test. Three of the subjects (V. J. H.; D. L. S.; A. S. C.) had not known of any glycosuria before the test. L. J. H. was known to show sugar in the urine and had a familial history of diabetes, though no symptoms were present. C. E. D. had sometimes shown glycosuria after meals and sometimes was entirely free; there were, however, no symptoms and no history, personal or familial, of diabetes. All had normal fasting blood-sugars.

Table IV. *Afternoon glycosuria.*

	Fermentable sugar every 2 hours in subjects				
Time	V. J. H. mg.	D. L. S. mg.	A. S. C. mg.	L. J. H. mg.	C. E. D. mg.
7–9 a.m.	0	0	0	0	0
*9–11 ,,	0	0	0	7	22
11–1 p.m.	0	0	11	7	63
†1–3 ,,	0	0	7	39	23
3–5 ,,	0	0	9	7	8
‡5–7 ,,	132	0	0	174	277
§7–7 a.m.	0	0	0	—	0

* Breakfast + 50 g. glucose. † Lunch *ad lib.* ‡ 50 g. glucose. § Dinner *ad lib.*

The results are shown in Table IV. The two subjects known to show glycosuria excrete recognisable fermentable sugar after the breakfast. One has a few mg. of fermentable sugar during the middle part of the day only. The

remaining two excrete none. The contention of Benedict is evidently not universally applicable. 50 g. of glucose taken with breakfast will not ensure a glycosuria. Nevertheless in view of our own experience with glucose in the afternoon it is possible that his contention may be true of a larger number of people than our few examples would indicate.

Of great interest is the fate of the glucose taken at 5 p.m. In two subjects it evidently had no effect but in the other three it produced a large excretion. Even in subjects L. J. H. and C. E. D. who showed fermentable sugar after breakfast, the excretion was much greater after the afternoon dose. Most interest, however, centres about V. J. H. because (except for the result of a previous similar experiment) it was the first time fermentable sugar had been found in his urine. The zero excretions of fermentable sugar throughout the rest of the day show this subject to be neither a renal glycosuriac, nor a cyclic hyperglycaemic. Previous tests had also shown a normal glucose tolerance curve.

We have termed this phenomenon of glycosuria after a dose of glucose in the afternoon, when it is absent after a fasting dose of glucose, or increased glycosuria in the afternoon when it is present in the morning test "afternoon glycosuria."

For a long time we were uncertain of the real nature of the phenomenon, being unable to control its appearance or disappearance. Thus under ordinary conditions of diet D. L. S. never showed a trace of fermentable urinary sugar, morning or afternoon. V. J. H. showed the phenomenon twice, and then it disappeared on ordinary diets. We consequently carried out a large number of experiments on these subjects in an endeavour to trace some particular influence of the noon meal. These, though they proved failures from our immediate standpoint, led to another possible source of fermentable urinary sugar. Finally by using a new series of subjects, we were able to convince ourselves that afternoon glycosuria is a common occurrence and may represent the concomitant of a regular rhythm in the glucose tolerance.

Table V. *Showing excretion of fermentable sugar 2–4 p.m. and glycosuria 4–6 p.m. after 50 g. glucose at 4 p.m.*

No.	2–4 p.m. mg.	4–6 p.m. mg.	No.	2–4 p.m. mg.	4–6 p.m. mg.
1	12	0	10	2	90
2	19	23	11	0	0
3	12	9	12	12	6
4	0	6	13	6	76
5	0	0	14	0	7
6	26	56	15	0	4
7	0	0	16	6	0
8	0	5	17	6	86
9	4	10	18	6	25

In Table V are shown the fermentable sugar excretions of the 18 subjects of Table II, during the hours 2–4 p.m. and 4–6 p.m. Lunch (*ad lib.*) was to be taken at noon but we cannot be certain that this condition was kept absolutely.

At 4 p.m. they arrived at the laboratory and a glucose tolerance test with 50 g. glucose was carried out, similar to the usual morning one. In view of the claims of Lennox [1927, 1, 2] that the second glucose tolerance test always gives lower blood-sugar levels than the first, half our subjects received the morning test first, and half received the afternoon test first. A fortnight was allowed to elapse between the 2 tests on the same subject. The 2–4 p.m. specimens often showed significant amounts of fermentable sugar. In view of the uncertainty of the time of the midday meal, this may represent post-prandial sugar similar to the subjects of Table V. The significant results are 4–6 p.m. when 13 out of 18 show fermentable sugar. This under the circumstances can only be glucose. This is in sharp contrast with the results in Table II when only 1 out of 18 showed unmistakably a little glucose.

Blood-sugar curves at 4 p.m.

In Figs. 1 and 2 are shown the results of the blood-sugar determinations of the 18 subjects whose urinary fermentable sugars are reported in Tables II and V. There is one outstanding difference between the two sets of curves. In the afternoon series the peak of the curve is always higher than in the corresponding morning curve. In a few of the subjects the difference is so small that it could be counted as an experimental error, except that it is always in the same direction. In others, however, the difference is so marked that the results cannot be doubted. In 14 out of the 18 the difference is 20 mg./100 cc. or over. It seems evident to us that the higher peak of the afternoon curves represents a change in the tolerance of the individual towards sugar, either as a result of the day's activities, or as a sequence to the taking of food. It represents a change towards the condition designated as "cyclic hyperglycaemia" by the Scandinavian clinical workers or the "lag type curve" by the English. Some of the curves fulfil the criteria of "lag type" as defined by MacLean. There thus appears to be a greater chance of exceeding the renal threshold for sugar in the afternoon than under the usual fasting morning conditions of a glucose tolerance test. If the average figures for the peaks of our morning and afternoon curves are compared they are 159 and 191 mg./100 cc. glucose respectively. The average difference is not large, but at the peak of the curve it may be quite sufficient to be the cause of a glycosuria in the afternoon when none is present in the morning. It is to be noted that post-alimentary hypoglycaemia occurs in half the afternoon curves.

Are the results with glucose applicable to the occurrence of fermentable sugar in post-prandial urines? We believe they may be. Increases in blood-sugar are not obtained with glucose only. Bread [Jacobsen, 1913], potatoes [MacLean and De Wesselow, 1921; Rosenthal and Ziegler, 1929], rice [Kageura, 1922; Sakaguchi, Matsuyama and Watanabe, 1922], cooked starch [Foster, 1923], mixed meals [Kjer, 1924], have been used to study carbohydrate tolerance by means of blood-sugar findings. In general they give the same type of blood-sugar curve as glucose. The blood-sugar curve may remain below

the renal threshold for sugar, or may exceed it. Jacobsen [1913], using the Bang method of sugar analysis on finger blood, gave 157 g. of white bread to normal individuals and showed a rise in blood-sugar, sometimes to such an extent above the threshold that clinical glycosuria was evident. The test was given, sometimes fasting and sometimes $1\frac{1}{2}$ hours after a light breakfast. There is thus no reason to exclude glucose as a possible post-prandial urinary fermentable sugar. At any rate it is certain from our experiments that the absence of glucose from the urine after its oral administration under fasting conditions in the morning is no argument for its non-appearance at other times during the day.

Our findings are against the conclusion drawn by many workers that the glucose tolerance curve is a constant for the individual [Hansen, 1923; Neilsen, 1928]. Such a conclusion we believe to be approximately correct for fasting conditions. Our morning tolerance curves show little inclination to rise above the usual threshold value. Under the shifting conditions of daily work and food, however, they vary. Moreover, a great deal of the work on which the idea of the constancy of the glucose tolerance test is based is itself open to criticism owing to the use of venous blood (now well known to give much lower peaks at the height of glucose ingestion [Friedenson, *et al.* 1928]), the collection of too infrequent blood samples to catch the peak blood-sugar value (even our 15 min. samples are barely sufficient), and to the use of relatively inexact clinical methods of detecting the glycosuria instead of methods of delicacy equal to those used in the blood analyses. This last reason has probably been a most potent factor in the lack of recognition of this variation in the glucose tolerance curve. All our urines were negative to Benedict's qualitative sugar reagent. With 30-minute blood samples probably only in 5 out of our 18 cases would there be sufficient difference in the afternoon curves to justify a differentiation from the morning curves. This general agreement, coupled with the negative Benedict test in the urine, would be sufficient to label all curves as normal in type, and the majority as approximately constant for the individual.

It might be objected that our results are brought about by the nature of the preceding meal or diet. Our subjects, however, were on their usual diet, and no restriction was placed on the nature of their noon meal. We obtained from each subject a list of the articles of food making up his lunch on the day of the afternoon test. They were very varied. The utmost that can be said is that the lunch was usually a light one. We have, however, precise experiments on the influence of the noon meal on the afternoon glucose tolerance test.

The nature of the noon meal and its effect on 4 p.m. glucose tolerance curves.

There has been a considerable amount of investigation on the effect of the previous diet on the ordinary glucose tolerance test as shown in blood-sugar curves. The most pronounced effect is that of fat contrasted with carbohydrate. High fat—low carbohydrate diets have been reported by Southwood [1923], Greenwald, Gross and Samet [1924], Sweeney [1927], Stenstrom [1927], Odin

[1927], Kohn, Fries and Felshin [1927], Malmros [1928] and Tolstoi [1929] to decrease the tolerance. Starvation [Bang, 1913; Traugott, 1922; Sevringhous, 1925; Pemberton and Foster, 1920; Du Vigneaud and Karr, 1925; Titiso, 1926; Harding and Van Nostrand, 1929; Hines, Boyd and Leise, 1929] where the body lives mainly at the expense of the fat depôts shows similar blood-sugar curves after glucose ingestion. High carbohydrate diets, conversely, increase the tolerance. The effect of protein is not so clear, Kageura [1922] had reported it to decrease the tolerance but Greenwald, Gross and Samet [1924], on an analysis of his diet, concluded that the effect was due to fat. Heinbecker [1928] found the glucose tolerance curves of the Greenland Eskimo living on a diet high in protein and fat to be normal. Sweeney [1927] found blood-sugar curves after 2 days' protein feeding to medical students intermediate in character between those obtained after a similar period of fat or carbohydrate feeding. On general grounds it might thus be expected that the character of the noon meal would influence the glucose tolerance test at 4 p.m. The only doubts that might exist would be the short time available for the effect of the meal, and whether one meal, however distinctive in character, would be sufficient to offset the influence of a previous ordinary diet. The shortest time in which a characteristic diet has been shown to affect a glucose tolerance test is recorded by Malmros who observed the change produced by a high fat diet for 1 day. We adopted 3 characteristic noon meals[1].

(1) *High fat meal:* 130 g. of 50 % cream made up partly as a cream soup, partly as whipped cream flavoured with saccharine, and partly taken in coffee.

(2) *High carbohydrate meal:* 150 g. carbohydrate in the form of potatoes, sweet potatoes, celery, lettuce, beets, carrots, tomatoes, and bread.

(3) *High protein meal:* 100 g. of protein in the form of roast veal and egg-white.

The subjects were laboratory workers; the noon meal was taken precisely at 12 noon; 2-hour urine specimens were collected commencing at 10 a.m. and the glucose tolerance test (50 g.) was commenced precisely at 4 p.m. An ordinary morning glucose tolerance test was performed on the subjects. A fortnight elapsed between each test. The fermentable urinary sugars are reported in Table VI, and the cutaneous blood-sugar curves on 6 of the subjects in Fig. 3. Venous blood-sugar curves on the remaining 2 subjects showed similar results but are not reported.

The urines show an almost remarkable paucity of positive results exclusive of the 4–6 p.m. period. None of the subjects shows fermentable sugar between meals. None is to be reckoned as a renal glycosuriac. It is not to be expected that a glycosuria should follow the high fat meal. No worker as far as we are aware has observed hyperglycaemia after fat. After protein or amino-acid feeding, however, rises in the blood-sugar have been noted. Cammidge, Forsythe

[1] The authors wish to express their thanks to Miss M. J. Porter and Miss L. Bryant of the Diet Kitchen of the Toronto General Hospital for their help, their unfailing courtesy, and the ingenuity with which they prepared the meals.

and Howard [1921] and Petren [1923] have noted hyperglycaemia after protein feeding. Pollak [1922] and Schatti [1923] found increased blood-sugar after amino-acids. Folin and Berglund, using venous blood for analysis and Lundsgaard [1930, 1, 2], on capillary blood, failed to find any increase in sugar either in man or in dogs after amino-acid or protein feeding. Rapport [1930] has recently reviewed this question from the general standpoint of the interconversion of foodstuffs. Our results, however, show no effect upon the urine in the form of fermentable sugar. It is after the high carbohydrate meal, however, that to us, the paucity of results possesses a possible significance. Only

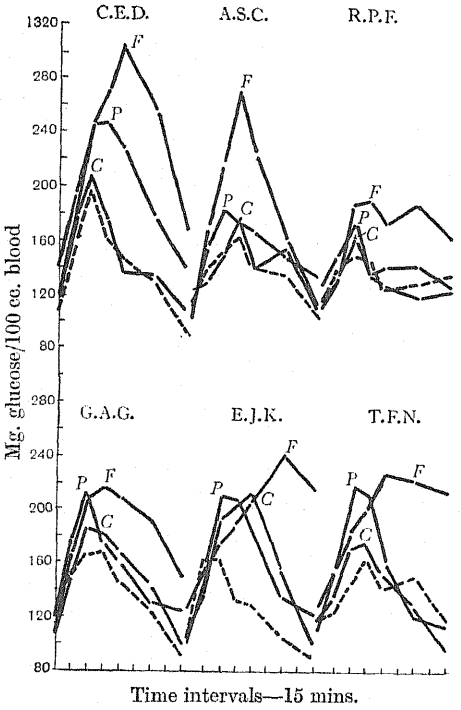


Fig. 3. Showing morning and afternoon tolerance curves after 50 g. glucose, with a variation in the nature of the noon meal.

Morning curves ------- Afternoon curves ———

C = Noon meal of carbohydrate. *P* = Noon meal of protein. *F* = Noon meal of fat.

2 out of 8 show glycosuria in the 12–2 p.m. specimen. In both of these the amount is considerable. In Table III the series of post-prandial urines shows 50 % of fermentable sugars. The amounts however are not large. In Table IV after mixed meals the subjects who show fermentable sugar show small or moderate amounts at hours other than immediately after meals. In Table V also a considerable percentage of subjects show small amounts of fermentable sugar. Our high carbohydrate noon meal contains 150 g. of carbohydrate almost exclusively in the form of starch. There is no cane sugar in the meal except a very small amount left after boiling the beets, carrots and sweet

Table VI. *Fermentable sugar in 2-hour urine specimen.*

Time	C. E. D. Noon meal of C.	C. E. D. Noon meal of P.	C. E. D. Noon meal of F.	A. S. C. Noon meal of C.	A. S. C. Noon meal of P.	A. S. C. Noon meal of F.	R. P. F. Noon meal of C.	R. P. F. Noon meal of P.	R. P. F. Noon meal of F.	G. A. G. Noon meal of C.	G. A. G. Noon meal of P.	G. A. G. Noon meal of F.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
10 a.m.–12 noon	0	0	0	0	0	0	0	0	0	0	0	0
*12 noon–2 p.m.	141	0	0	0	0	0	0	0	0	0	0	0
2 p.m.–4 p.m.	0	0	0	0	0	0	0	0	0	0	0	0
†4 p.m.–6 p.m.	39	223	1696	12	16	275	0	5	0	8	34	317
Morning glucose tolerance test showed	6 mg.			0 mg.			0 mg.			10 mg.		

Time	E. J. K. Noon meal of C.	E. J. K. Noon meal of P.	E. J. K. Noon meal of F.	T. F. N. Noon meal of C.	T. F. N. Noon meal of P.	T. F. N. Noon meal of F.	V. J. H. Noon meal of C.	V. J. H. Noon meal of P.	V. J. H. Noon meal of F.	D. L. S. Noon meal of C.	D. L. S. Noon meal of P.	D. L. S. Noon meal of F.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
10 a.m.–12 noon	0	0	0	0	0	0	0	0	0	0	0	0
*12 noon–2 p.m.	168	0	0	0	0	0	0	0	0	0	0	0
2 p.m.–4 p.m.	0	0	0	0	0	0	0	0	0	0	0	0
†4 p.m.–6 p.m.	80	67	479	0	42	367	0	131	738	0	10	0
Morning glucose tolerance test showed	0 mg.			0 mg.			0 mg.			0 mg.		

* Noon meal. † 50 g. glucose. C. = High carbohydrate. P. = High protein. F. = High fat.

potatoes. The fermentable sugar occurring in C. E. D. and E. J. K. after the high carbohydrate lunch must then almost certainly be glucose.

E. J. K. however shows a morning blood-sugar curve allowing the escape of no glucose, and illustrates our contention that the behaviour of the morning glucose tolerance curve is not an exact criterion of the action of carbohydrate at a meal later in the day. In contrast, G. A. G. shows 10 mg. glucose in the morning test and none after the carbohydrate meal. In general, however, the contrast we have drawn between the results shown in Table VI and other post-prandial results gives rise to the suspicion that fermentable sugar other than glucose can occur after meals.

The results of Table VI show afternoon glycosuria in 18 out of 24 experiments. Generally, it appears to be greatest after the fat and least after the carbohydrate noon meal. Two subjects (R. P. F., D. L. S.) show it only after protein, and it seems desirable to emphasise the fact that all the subjects have shown afternoon glycosuria following the noon meal of protein. The blood-sugar curves of Fig. 3 show in all 6 subjects a higher peak in the afternoon than the morning. Of the afternoon curves, those following the carbohydrate noon meal are the lowest, though in E. J. K. and A. S. C. the difference between the peak after carbohydrate and that after protein is very small. The results on the day of the high fat meal are of extreme interest. Three of the tolerance curves are almost diabetic in type. Only in 1 does the sugar value regain its initial value in 2 hours. There is thus not only a high peak but a prolongation in the tolerance curve. The 6 subjects who showed glycosuria gave a positive Benedict sugar test. These same 6 subjects showed marked ketonuria by Rothera's and Gerhardt's tests. It may perhaps be of significance that the 2 subjects (R. P. F. and D. L. S.) who showed no ketonuria also showed no glycosuria.

Malmros [1928] concludes that acidosis is not fundamentally related to the occurrence of glycosuria in non-diabetics but that it may be of importance as a supplementary factor. Our experiments are too few to throw much further light on this problem, but we should like to reserve this aspect of experimental ketonuria for future investigation.

The effect of protein appears intermediate between carbohydrate and fat. An interesting side-issue is the blood-sugar value at 4 p.m. after protein compared with its corresponding value after carbohydrate and fat. The figures are given in Table VII. With one exception, where the subject was continuously nauseated after the meal, the sugar values, both in cutaneous and venous blood were higher after protein than after carbohydrate or fat. In view of the results produced by individual amino-acids on blood-sugar levels in other animals this phenomenon may represent a stimulation of metabolism, rather than a new production of glucose. Lundsgaard [1930, 2] regards the hyperglycaemia from amino-acid feeding as a toxic effect. Also in view of the fact that our method of analysis determines the non-sugar reducing substances as well as true sugar, some uncertainty must attach itself to small differences.

Table VII. *Cutaneous blood-sugar at 4 p.m. after noon meal of protein, carbohydrate or fat.*

Mg. per 100 cc. blood.

Meal	C. E. D.	A. S. C.	R. P. F.	G. A. G.	E. J. K.	T. F. N.	V. J. H.*	D. L. S.*
Protein	140	111†	127	121	127	127	119	114
Carbohydrate	117	122	116	107	111	117	95	110
Fat	120	101	111	109	101	109	110	112

* Venous blood. † Nauseated.

There thus seems to be a good chance of a subject exhibiting glycosuria in response to 50 g. of glucose at 4 p.m. if the noon meal is of mixed character, and, under ordinary variations, the phenomenon will appear independently of the nature of the diet. Variations in individuals, however, are evidently to be expected. The reason for the disappearance of the phenomenon in V. J. H. noted above we now believe to have been an alteration in the glucose tolerance due to repeated experimental administration of glucose which is reported by several observers to improve the tolerance. Eisner [1926] fed glucose daily to rabbits and noted the production of a low type glucose tolerance curve. John [1922] gave 100 g. of glucose to two normal individuals on 5 successive days. The blood-sugar peak on the 1st day was 260 mg./100 cc. glucose; on the 5th day it was 90 mg./100 cc. Sansum, Blatherwick and Bowden [1926], Gibson [1929] and Rabinowitch [1930] have all recently reported the stimulating effect of glucose or carbohydrate feeding on the carbohydrate tolerance of diabetics. It seems very probable that continued administration of glucose may affect also the afternoon glucose tolerance curve. It was a recognition of this possibility that led the authors to select 18 medical students as an entirely new series of subjects for the demonstration of afternoon glycosuria as a common phenomenon.

We can find only a few odd references in the literature bearing on afternoon glycosuria and the corresponding blood-sugar curves. Staub [1921] states that if 20 g. glucose are given 5 hours after a meal, the blood-sugar curve is higher than after a similar dose taken 5 hours later again. Another possibility of rhythm in glucose tolerance may perhaps be found in the work of Kohn, Fries and Felshin [1927], who reported that 2 or 3 days after the change from a high fat diet to a high carbohydrate diet, the glucose tolerance test showed abnormally low blood-sugar curves, and only later gave curves of more normal altitude. This was found in children but has not been noticed by other observers working on adults. The phenomenon may perhaps also have occurred in some of the experiments of Folin and Berglund [1922, 2] for they remark that they "are under the impression that excessive hyperglycaemias are more easily obtained in the evenings than during the early forenoon hours."

Our observations on afternoon glycosuria and its cause apparently bring us into conflict with a widely held view that the carbohydrate tolerance, both of normals and diabetics is lowest in the morning. Naunyn is quoted by Folin and Berglund [1922, 2] as giving this statement as a general expression of clinical opinion on the tolerance of the diabetic in 1906. Peters and Van Slyke [1931] use the morning rise in blood-sugar in the diabetic as illustrating the necessity for the insulin dosage to be highest in the morning. The lowered tolerance of the severe diabetic in the morning may represent one of the differences between that disease and the normal. Such evidence as we have seen, as that of Page [1923], Jonas, Miller and Teller [1925] and Watson [1929], however, by no means inclines us to the view that the normal shows the same lowered morning tolerance. Nor do we wish to be put on record as stating that 4 p.m. blood-sugar tolerance curves are always to be found with a higher peak than those at 9 a.m. in normal individuals. We merely state that such is a common occurrence and leave any discrepancy between our results and those of other observers on normal carbohydrate tolerances to future investigation.

Objection may perhaps be raised to our description of some of the 4 p.m. blood-sugar curves as "lag type." The blood-sugar curves themselves fulfil the criteria of the "lag type" curve. The glycosuria which accompanies our curves, however, is minute compared with "clinical" glycosuria. This difference, however, is only one of degree and we believe may be due to the clinical observations being made on individuals possessing a renal threshold for sugar lower than the usual normal average. Thus a subject possessing a renal sugar threshold of 100–120 mg./100 cc. would show no sugar in the fasting urine with the ordinary clinical tests. After 50 g. glucose, with a usual rise of blood-sugar to 170–180 mg./100 cc. he might show a positive Benedict test. He would then be classed as a renal glycosuriac of the intermediate type described by Graham [1923]. Should his blood-sugar peak rise to 200–220 mg./100 cc. or over it is almost certain that urinary sugar would be found. He would then be classed as a "lag type." Although this may be the explanation of the clinical glycosuria accompanying the "lag type" curve, it does not explain why such

individuals show a "lag type" curve in the morning while our normals only show the high peak in the afternoon.

Urinary fermentable sugar after fruits, honey, orange juice and invert sugar.

In the preceding sections of this paper we have given cogent reasons why glucose may at times be a constituent of normal urine. At the same time doubts have been expressed on the single nature of the urinary fermentable sugar after meals under all conditions. These suspicions were engendered by observations on the authors in their preliminary search for conditions under which afternoon glycosuria might be demonstrable. They took a series of noon meals of varied character. The high fat, protein and carbohydrate, with their negative results, have already been discussed in connection with Table VI. In addition, the effect of variations in the proportions of protein, fat and carbohydrate were studied, using the same food materials. Entirely negative results were obtained. When, however, in high carbohydrate meals a large proportion of the carbohydrate arose from fruits, small amounts of fermentable sugar made their appearance in the urine. The details of some of the meals and the results of some of the experiments are shown in Table VIII. In all, 19 experiments of this nature were performed and fermentable urinary sugar was

Table VIII. *Excretion of fermentable sugar after meals containing large amounts of fruit.*

Expt.	Character of noon meal		Subject	Fermentable sugar 12 noon–2 p.m.	Fermentable sugar 2–4 p.m.
1	High protein and fat	(*a*)	V. J. H.	0	0
			D. L. S.	0	0
2	(*a*) + 60 g. cane sugar		V. J. H.	0	0
			D. L. S.	0	0
3	Mixed	(*b*)	V. J. H.	0	0
			D. L. S.	0	0
4	Mixed	(*c*)	V. J. H.	0	0
	Mixed	(*d*)	D. L. S.	0	0
5	Carbohydrate (fruit salad)	(*e*)	V. J. H.	24	5
			D. L. S.	12	8
6	Carbohydrate (fruits)	(*f*)	V. J. H.	15	0
			D. L. S.	25	0
18	Carbohydrate (fruits)	(*g*)	V. J. H.	18	0
			D. L. S.	8	11
19	Carbohydrate (fruits)	(*h*)	V. J. H.	30	0
			D. L. S.	?	0

(*a*) Oysters, sirloin steak, rolls, butter, coffee, cream, sugar.

(*b*) Lamb, potatoes, carrots, bread, butter, apple pie, tea, cream, sugar.

(*c*) Beef, potatoes, cheese, bread, butter, coffee, apple pie, jelly.

(*d*) Soup, veal, potatoes, corn, bread, butter, cream, coffee.

(*e*) Salad composed of potato, celery, apple, cherries, nuts, mayonnaise; bread, butter, apple pie, cheese, coffee, sugar.

(*f*) Grapefruit, prunes, apricot, peaches, bread, butter, apple pie, coffee, cream, sugar (50 g.).

(*g*) 150 g. potato, 80 g. carrot, 70 g. sweet potato, 120 g. orange (edible portion), 100 g. banana (edible portion), 200 g. orange juice, cream, coffee.

(*h*) Largely orange and banana, small amount potato and carrot, 200 g. orange juice.

clearly demonstrated only in 4, *viz.* when the meal contained a large amount of fruit. Since neither author is in the habit of showing demonstrable urinary fermentable sugar after ordinary meals, the positive results after fruit, in both authors, and occurring under the same conditions, become thus more remarkable and point to the occurrence of fermentable sugar in urine other than glucose.

We extended our observations to two other subjects; one of whom, judging by the results in Table VI, possesses as efficient a mechanism as D. L. S. for the retention of glucose. To these subjects we gave 500 cc. of orange juice at 9 a.m. under the conditions of a morning glucose tolerance test. The juice was made from 12 Florida oranges, extracted the previous afternoon, and kept overnight in a refrigerator. It represents 50 g. edible carbohydrate. The results are shown in Table IX. Both showed fermentable urinary sugar. A repetition of the experiment gave the same result. Honey, and 50 g. of invert sugar prepared in the laboratory by the action of 0·5 *N* HCl on cane sugar, also gave fermentable urinary sugar. Addition to the orange juice of (*a*) glucose, (*b*) cane sugar, (*c*) invert sugar did not increase the urinary fermentable sugar. 50 g. cane sugar gave negative results. In view of this result we observed the action of glucose which had been allowed to stand overnight with 0·5 *N* HCl, in case the action of the acid might produce small amounts of fermentable material which escaping into the urine would be reckoned as glucose, though it is more usual to suppose the stability of sugars under such conditions. The result of the experiment was negative.

Table IX. *Excretion of fermentable sugar after orange juice, honey and invert sugar.*

Expt.	Ingestion at 9 a.m. of	Subject	Fermentable sugar 7 a.m.–9 a.m.	9 a.m.–11 a.m.	11 a.m.–1 p.m.
			mg.	mg.	mg.
20	500 cc. orange juice	R. P. F.	0	7	0
		C. E. D.	0	7	0
21	500 cc. orange juice + 40 g. cane sugar	R. P. F.	0	12	0
		C. E. D.	0	12	0
22	90 g. cane sugar in 500 cc. water	R. P. F.	0	0	0
		C. E. D.	0	0	0
23	500 cc. orange juice	R. P. F.	—	5	0
		C. E. D.	0	15	0
24	500 cc. orange juice + 40 g. glucose	R. P. F.	0	13	0
		C. E. D.	0	7	0
25	500 cc. orange juice + 40 g. partly hydrolysed cane sugar	R. P. F.	0	18	6
		C. E. D.	0	28	0
26	500 cc. orange juice + 40 g. invert sugar	R. P. F.	0	0	0
		C. E. D.	0	15	0
27	90 g. invert sugar in 500 cc. water (50 cc. *N*/2 HCl)	R. P. F.	0	8	0
		C. E. D.	0	10	0
28	120 g. honey in 500 cc. water	R. P. F.	0	5	0
		C. E. D.	0	15	5
29	50 g. glucose + 50 cc. *N*/2 HCl in 500 cc. water	R. P. F.	0	0	0
		C. E. D.	0	0	0

Such a series of experiments could point only to the presence of free fructose, some decomposition product by acid, or a new substance removable by yeast and formed as a metabolic product from fructose. We feel our experiments may offer a partial explanation of some of the results in the literature on post-prandial sugar. The use of jams, preserves, and marmalade, where acid fruits are boiled with cane sugar, might offer another source of urinary fermentable sugar.

Urinary fermentable sugar after fructose.

On six subjects we examined the action of 50 or 25 g. of fructose both at 9 a.m. under the usual fasting conditions, and at 4 p.m. The fructose used was Schuchart's "puriss. cryst." ($[\alpha]_D^{20} - 92{\cdot}5°$). The urine findings are given in Table X and the capillary blood-sugars in Fig. 4.

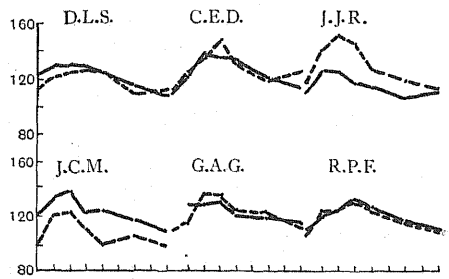


Fig. 4. Showing morning and afternoon tolerance curves after 50 or 25 g. of fructose.
Morning curves -------- Afternoon curves ————

All subjects showed fermentable urinary sugar both in the morning and afternoon. The amounts are small, and are of the same magnitude as those shown after fruits, *etc.* in the preceding section. Moreover in the morning experiments there is seen an excretion lasting 2–4 hours after the administration of the sugar in three of the six subjects. A similar continued excretion is noticed in some of the experiments after fruit, *etc.* and in some of the post-prandial sugars. We have referred to those in Table IV. Some of those in Table V may also represent a similar continued excretion. Recently Corley [1929] has shown the presence of small amounts of urinary fructose after fructose feeding in the rabbit. The presence of fructose in the blood-stream after fructose feeding has been shown in man by Folin and Berglund [1922, 2] using the Seliwanoff test, and by Corley in the rabbit using the modified Van Creveld method. There thus seems no reason to refuse to label the urinary fermentable sugar in our experiments after the feeding of fructose, fruits, honey or invert sugar, as fructose, unless some unidentified metabolic product behaves in a similar way. That fructose differs from glucose in its metabolism is well known. It possesses its own rate of absorption [Cori and Cori, 1925]. Its high ability to form glycogen [Cori, 1926], its poor utilisation in the muscles [Mann and Bollman,

1930] and the accompanying formation of lactic acid after oral administration [Campbell and Maltby, 1928] all mark it off from glucose.

Table X. *Urinary fermentable sugar in six subjects after oral administration of fructose at 9 a.m. and at 4 p.m.*

Subject	Fructose ingested	Fermentable sugar in 2 hr. urine: Fructose at 9 a.m. 9–11	Fructose at 9 a.m. 11–1	Fructose at 4 p.m. 4–6
	g.	mg.	mg.	mg.
D. L. S.	50	7	4	8
C. E. D.	50	7	0	8
J. J. R.	25	21	0	13
J. C. M.	25	14	0	13
G. A. G.	50	19	10	13
R. P. F.	50	8	0	9

The differentiation from glucose is also to be noticed in the shape of the blood-sugar curves after ingestion. The total blood-sugar is only elevated a little in comparison with the rises noted after glucose. The morning curves shown in Fig. 4 confirm many observations in the literature. The afternoon curves are almost exactly similar to the morning curves. There is no evidence of any afternoon fructosuria either from the urinary findings, or from any increased peak or height in the blood-sugar curves. This again differentiates its metabolism from glucose. At present we do not wish to offer any explanation, either of the phenomenon of afternoon glycosuria or of our failure to observe a similar phenomenon after fructose.

General conclusions.

1. Within the limits of the analytical methods fermentable sugar is absent from normal fasting urine. The possible amounts of fermentable sugar must be under 5 mg./100 cc. and are more likely to be less than 2–3 mg./100 cc. We have excluded known renal glycosuriacs from our observations.

2. After glucose ingestion in the morning under fasting conditions no increased amount of fermentable sugar can be detected in the urine. A survey of a large number of individuals will, however, undoubtedly reveal some showing glycosuria, though too small to be detected by the usual clinical reagents. The percentage in which such individuals occur in a series will influence the experimenter to declare the presence or absence of glucose in normal urine after glucose ingestion. Three such examples occur in our experiments. In one there appears to be a slightly lowered renal threshold.

3. 50 % of medical students show small amounts of fermentable sugar in the post-prandial afternoon urine.

4. Two subjects who showed no fermentable urinary sugar, fasting, after glucose in the morning, or after ordinary mixed meals, showed fermentable sugar after large amounts of fruit.

5. Fermentable urinary sugar was also shown to occur in two other subjects after orange juice, honey and invert sugar, taken fasting.

6. 50 or 25 g. of pure fructose given as a fructose tolerance test in the morning or in the afternoon at 4 p.m. give rise to urinary fermentable sugar.

7. 50 g. glucose at 4 p.m. give rise to glycosuria in individuals showing none under the fasting conditions of the morning test. This phenomenon of afternoon glycosuria is of frequent occurrence. It is least likely to occur if the noon meal is high in carbohydrate. It has always occurred if the noon meal is high in protein. A high fat noon meal is also very likely to cause afternoon glycosuria.

8. The blood-sugar curves at 4 p.m. taken under ordinary conditions of diet all possess a higher peak than the corresponding fasting morning tolerance curves. The peak often rises above the usual renal threshold for sugar. This accounts for afternoon glycosuria. Some of the blood-sugar curves resemble those of the MacLean "lag type."

9. The nature of the noon meal affects the blood-sugar curve after 50 g. glucose at 4 p.m. A high carbohydrate meal gives rise to the lowest curve. A high fat meal gives rise to a high peak curve, sometimes remaining high at the end of 2 hours. Ketosis was observed in six out of eight experiments after our high fat noon meal. A high protein noon meal gives a high peak blood-sugar curve. All tolerance curves at 4 p.m. are higher than the morning tolerance curve in the same individual.

10. We have found no phenomenon corresponding to afternoon glycosuria in the urine, or in the shape of the blood-sugar curves, after the oral administration of fructose. This may serve as additional evidence differentiating the metabolism of glucose from that of fructose.

11. The non-occurrence of glycosuria after the usual morning glucose tolerance test cannot be taken as a criterion of its continued non-occurrence throughout the day. After a noon meal where the carbohydrate consisted almost entirely of starch a subject showed glycosuria, although a morning tolerance test with glucose was entirely negative. Another subject showed a few mg. of glucose in the urine after the morning test, but none after the carbohydrate meal.

12. The occurrence of fermentable sugar in normal urine is a variable phenomenon depending on a number of complex factors.

REFERENCES.

Bang (1913). Der Blutzucker. (Wiesbaden.)
Benedict and Osterberg (1923). *J. Biol. Chem.* **55**, 769.
—— —— and Neuwirth (1918). *J. Biol. Chem.* **34**, 217.
Cammidge, Forsythe and Howard (1921). *Brit. Med. J.* ii, 586.
Campbell and Maltby (1928). *J. Clin. Invest.* **6**, 303.
Cori (1926). *Proc. Soc. Exp. Biol. Med.* **23**, 459.
—— and Cori (1925). *J. Biol. Chem.* **66**, 691.

Corley (1929). *J. Biol. Chem.* **81**, 81.
Du Vigneaud and Karr (1925). *J. Biol. Chem.* **66**, 281.
Eagle (1927). *J. Biol. Chem.* **71**, 481.
Eisner (1926). *Z. ges. exp. Med.* **52**, 214.
Faber (1926). *J. Clin. Invest.* **3**, 203.
Finley and Rabinowitch (1924). *Quart. J. Med.* **67**, 260.
Folin and Berglund (1922, 1). *J. Biol. Chem.* **51**, 209.
—— —— (1922, 2). *J. Biol. Chem.* **51**, 213.
—— and Svedberg (1926). *J. Biol. Chem.* **70**, 405.
Foster (1923). *J. Biol. Chem.* **55**, 291, 303.
Friedenson, Rosenbaum, Thalheimer and Peters (1928). *J. Biol. Chem.* **80**, 269.
Geelmuyden (1915). *Morsk. Mag. Laegevidenskaben*, **13**, 985.
Gibson (1929). *J. Lab. Clin. Med.* **14**, 597.
Goto (1918). *Arch. Int. Med.* **22**, 96.
—— and Kuno (1921). *Arch. Int. Med.* **27**, 224.
Graham (1923). *Quart. J. Med.* **63**, 236.
Gray (1923). *Arch. Int. Med.* **31**, 241.
Greenwald, Gross and Maguire (1927). *J. Biol. Chem.* **75**, 491.
—— —— and Samet (1924). *J. Biol. Chem.* **62**, 401.
Hamilton (1928). *J. Biol. Chem.* **78**, 63.
Hansen (1923). *Acta Med. Scand.* **58**, 585.
Harding and Van Nostrand (1929). *Trans. Roy. Soc. Can.* **23**, 293.
—— —— (1930). *J. Biol. Chem.* **85**, 765.
Hassan (1928) *Biochem. J.* **22**, 1332.
Heinbecker (1928). *J. Biol. Chem.* **80**, 461.
Hiller, Linder and Van Slyke (1925). *J. Biol. Chem.* **64**, 625.
Hines, Boyd and Leise (1929). *Amer. J. Physiol.* **88**, 240.
Holst (1925). *Acta Med. Scand.* **63**, 47.
Höst (1923). *J. Metabol. Res.* **4**, 315.
Jacobsen (1913). *Biochem. Z.* **56**, 471.
John (1922). *J. Metabol. Res.* **1**, 497.
Jonas, Miller and Teller (1925). *Arch. Int. Med.* **35**, 289.
Kageura (1922). *J. Biochem.* **1**, 333.
Kjer (1924). *Acta Med. Scand.* **61**, 159.
Kohn, Fries and Felshin (1927). *Amer. J. Dis. Child.* **34**, 857.
Lennox (1927, 1). *J. Biol. Chem.* **73**, 237.
—— (1927, 2). *J. Clin. Invest.* **4**, 331.
Linder, Hiller and Van Slyke (1924). *J. Clin. Invest.* **1**, 247.
Lund and Wolf (1926). *Biochem. J.* **20**, 259.
Lundsgaard (1930, 1). *Biochem. Z.* **217**, 125.
—— (1930, 2). *Biochem. Z.* **217**, 147.
MacLean (1924). Glycosuria and diabetes. (Constable and Co., London.)
—— and De Wesselow (1921). *Quart. J. Med.* **14**, 103.
Mann and Bollman (1930). *Amer. J. Physiol.* **93**, 671.
Malmros (1928). *Acta Med. Scand. Suppl.* 27.
Marsh (1921). *Arch. Int. Med.* **28**, 54.
Neilsen (1928). *Biochem. J.* **22**, 1490.
Odin (1927). *Acta Med. Scand. Suppl.* 18.
Page (1923). *J. Lab. Clin. Med.* **8**, 631.
Patterson (1926). *Biochem. J.* **20**, 43.
Pemberton and Foster (1920). *Arch. Int. Med.* **25**, 243.
Peters and Van Slyke (1931). Quantitative clinical chemistry, p. 132. (Williams and Wilkins Co., Baltimore.)
Peterson and West (1929). *Amer. J. Physiol.* **90**, 470.
Petren (1923). *Arch. exp. Path. Pharm.* **99**, 52.
Pollak (1922). *Biochem. Z.* **127**, 120.
Rabinowitch (1930). *Can. Med. Assoc. J.* **23**, 497.
Rapport (1930). *Physiol. Rev.* **10**, 349.

Raymond and Blanco (1928). *J. Biol. Chem.* **79**, 649.
Rosenthal and Ziegler (1929). *Arch. Int. Med.* **44**, 344.
Sakaguchi, Matsuyama and Watanabe (1922). *J. Biochem.* **1**, 371.
Sansum, Blatherwick and Bowden (1926). *J. Amer. Med. Assoc.* **86**, 178.
Schatti (1923). *Biochem. Z.* **143**, 201.
Sevringhous (1925). *J. Biol. Chem.* **63**, xlviii.
Somogyi (1926). *J. Biol. Chem.* **70**, 599.
—— (1927). *J. Biol. Chem.* **75**, 33.
Southwood (1923). *Med. J. Austr.* **10**, pt. 2, 460.
Staub (1921). *Biochem. Z.* **118**, 93.
Stenstrom (1927). *Zentr. inn. Med.* **48**, 244.
Sweeney (1927). *Arch. Int. Med.* **40**, 818.
Titiso (1926). *Trans. Roy. Soc. Can.* **20**, 33.
Tolstoi (1929). *J. Biol. Chem.* **83**, 747.
Traugott (1922). *Klin. Woch.* **1**, 892.
Van Creveld (1927). *Klin. Woch.* **6**, 697.
Van Slyke and Hawkins (1929). *J. Biol. Chem.* **83**, 51.
Watson (1929). *J. Lab. Clin. Med.* **15**, 234.

CXCVII. A MODIFICATION OF THE METHOD OF STEWART AND WHITE FOR THE DETERMINATION OF BLOOD-FAT, WITH OBSERVATIONS ON SEVERAL SPECIES IN POSTABSORPTIVE CONDITIONS.

By HAROLD EDWIN HIMWICH, HILDA FRIEDMAN[1] and MARY AGNES SPIERS.

From the Department of Physiology, Yale University, New Haven, Connecticut.

(*Received June 18th, 1931.*)

The present report contains a study of the method of Stewart and White [1925] for the determination of blood-fat, and full details are given concerning a modification which, in our hands, has proved more practicable than their original procedure. Substances interfering with the determination have been examined and the error of the method has been determined. The data also include observations on the fluctuations occurring in a given animal and the variations in the level of blood-fat of a series of animals of several species in the postabsorptive state.

Procedure.

Preparation. 5 cc. samples of oxalated blood are centrifuged for half an hour and 1 cc. of the plasma thus obtained is added to 35 cc. of an alcohol-ether mixture (3 parts 95 % alcohol—1 part ordinary ether). By adding the plasma drop by drop while constantly rotating the flask, a finely divided precipitate is obtained.

Extraction. The flask is plunged in a bath of boiling water. The mixture is boiled for a few seconds, cooled to room temperature and filtered into a 50 cc. volumetric flask. If the fat content of the blood is high, the accuracy is increased by making the alcohol-ether extract up to 100 cc. instead of 50 cc.

Saponification. Aliquot portions (15 cc.) of the filtrate are measured into 50 cc. Erlenmeyer flasks and controls are made on the same volume of alcohol-ether mixture. The mixture is evaporated to about 1 cc., then 5 cc. of 0·1 *N* NaOH are added from a burette and the mixture is again evaporated to approximately 1 cc. In order to complete the saponification, 5 cc. of absolute alcohol are added, and evaporation is continued until 2 cc. of the solution remain. A hot plate is always used for evaporation.

[1] Some of the data of this paper are taken from an essay presented by Hilda Friedman in candidacy for the degree of Master of Science, Yale University, 1928.

Acidification. Liberation of fatty acids. 5 cc. of 0·1 *N* HCl are added. This should exactly neutralise the alkali previously combined and liberate the fatty acids. The mixture is then boiled down to half its volume in order to expel any absorbed CO_2.

Titration. After adding 10 cc. of absolute alcohol, the solution is heated and titrated while hot with 0·02 *N* NaOH, using phenolphthalein as indicator.

Calculation. If 1 cc. of plasma is dissolved in 50 cc. alcohol-ether mixture and 15 cc. of the extract are used, the final aliquot corresponds to $1/50 \times 15$ or 0·3 cc. of plasma. With a final titration value for 0·02 *N* NaOH of X, the amount of fat present in 100 cc. of plasma is equal to $X \times 100/0{\cdot}3 \times 5{\cdot}38$; 5·38 being the factor when calculating in terms of tripalmitin. In a determination of fat, the titration value of the control is subtracted from that of the unknown and the difference is multiplied by the factor 1793 in order to determine the fat present in the blood sample in mg./100 cc. The result so obtained is then corrected for any change in concentration.

Recovery of the higher fatty acids. Accurately weighed amounts of pure palmitic and oleic acids were dissolved in definite volumes of the alcohol-ether mixture and determinations were made on portions of these solutions. The procedure for determining these samples was similar to directions given above for the treatment of plasma. The recovery of these fatty acids is given in Table I.

Table I. *Recovery of known amounts of fatty acids.*

Fatty acid	Amount present mg./100 cc.	Amount recovered mg./100 cc.	% recovered	Deviation of recovery
Palmitic	340	337·9	99·4	−0·6
Palmitic	888	900·0	101·0	+0·1
Palmitic	622	611·0	98·2	−1·8
Oleic	340	315·8	92·5	−7·5
Oleic	240	236·0	98·3	−1·7
Mixture, oleic + palmitic	410	406·0	98·0	−2·0
Mixture, plasma extract + oleic + palmitic	376	400·0	106·0	+6·0
			Average	±2·52

Interfering substances. A number of observations were made to determine whether lactic or butyric acid, or their sodium salts, or acetone acted as interfering substances in the estimation of fat in the blood. 1 cc. of solution containing 1·2 mg. of lactic acid was dissolved in the alcohol-ether mixture and made up to 50 cc. 1 cc. of solution containing 0·96 mg. butyric acid was similarly treated. Determinations were made on 15 cc. aliquots of these solutions using the same procedure as in the treatment of plasma. Similar determinations were made using sodium lactate, and butyrate, and acetone. None of these substances could be titrated by this method.

Constancy of recovery. It seemed advisable to estimate the change that might occur in blood drawn at short intervals of 2 minutes, and then again at intervals of 2 hours. Results have shown that blood obtained from dogs anaesthetised with amytal and in the postabsorptive state at intervals of

2 minutes and determined separately remained practically unchanged, the average deviation of a single determination being ± 0·4 %. The values of fat in blood taken at 2-hourly intervals for 8 hours, in the case of three such dogs, have shown that there is an average deviation of ± 2·2 %. In the case of unanaesthetised dogs the average deviation of a single determination was ± 3·5 %. Four of these determinations were corrected for total solids and yielded an average deviation of ± 3·7 % after the correction was made (average percentage deviation = ± 3·6). Repeated observations of the same animals over a period of 6 months are presented in Table II.

Table II. *Repeated observations on the same animal.*

Dog	Number of observations	Average fat value mg./100 cc.	Average % deviation
I	7	663	±15·30
II	7	692	±15·00
III	4	720	±20·60
IV	4	656	±13·00
V	2	767	±13·25
VI	2	675	±32·00
VII	2	691	± 2·91

Values under postabsorptive conditions. The average fat values for normal resting animals under postabsorptive conditions have been calculated from a large number of determinations and are as follows: dog 673, cat 611, monkey 501, rabbit 498, man 481 mg./100 cc. respectively.

Discussion.

The changes from the technique of Stewart and White. The method described above differs in some respects from that originally presented by Stewart and White [1925]. In the first place, we have found it possible to get satisfactory results using 1 cc. of whole blood or 1 cc. of blood-plasma instead of 2 cc. as indicated by Stewart and White [1925]; thus less blood is required. Determinations have been made of the fat content of 1 cc. of whole blood and 1 cc. of plasma from the same blood sample. In some instances there has been a difference between the fat content of the whole blood and that of the plasma. Therefore, blood-plasma instead of whole blood was used whenever possible, since the red blood corpuscles may vary in the course of an experiment and thus yield an erroneous result.

According to Stewart and White [1925], the alcohol-ether extract of plasma is brought to the boil and made up to volume. In order to withdraw the supernatant fluid, the mixture is allowed to stand so that the precipitate may settle. There are several objections to this procedure. Frequently the supernatant fluid is not entirely clear. Again, the fluid withdrawal may not be uncontaminated with the precipitate. Finally, the amount of filtrate obtained is limited by this method of withdrawal. For these reasons, filtration was substituted for decantation as described above. The extraction of lipins with filtering was practically complete because plasma was used. To quote Bloor

[1928], "the statement appearing in the literature and attributed to the author that the alcohol-ether extraction is not complete by this procedure (filtering) applies only to whole blood or corpuscles and not to plasma." A hot plate is preferable to a steam-bath for boiling down the various mixtures after each step in the process.

In the final step, according to Stewart and White [1925], 2 cc. of the final solution (composed of fatty acids and 10 cc. absolute alcohol) is equivalent to 1/10 of the original sample and is transferred and titrated with 0·1 *N* NaOH from a Rehberg burette. In the present modification of the method the total final solution is used for the titration. This avoids any error of transference.

In our analyses a micro-burette, with a very fine tip, and a capacity of 1·5 cc. is used. This burette is graduated in 0·01 cc. divisions, so that it is possible to interpolate to 0·001 cc. Checks within 0·005 cc. are frequently obtained.

It is found that on titrating in the manner suggested by Stewart and White [1925], with CO_2-free alkali, the CO_2 in the air interferes to such an extent as to give uncertain end points. The titration was therefore conducted at the boiling-point as described above. Furthermore, the absolute alcohol itself is so acid as to require almost as much of the alkali for its neutralisation as is necessary for an equal volume of fatty acid solution with the small quantities measured by Stewart and White [1925]. Attempts to rid the alcohol of its acidity by distilling over lime and caustic alkali were unsuccessful. The error due to alcohol is eliminated by the use of control determinations on the reagents.

As larger amounts of the more dilute alkali are used, the error of titration may be lessened. Therefore, 0·1 *N* NaOH was replaced by 0·02 *N* NaOH.

It became necessary to make a correction for changes in concentration of successive samples of plasma from the same animal, for the fatty acids determined by this method are present in the plasma as esters and chiefly in suspension in the form of triglycerides, only a small portion existing in solution as phospholipins [Bloor, 1916, 1, 2; Boyd, 1931]. This correction was made by drying a 1 cc. portion of each sample of plasma to constant weight at 100°. Thus it was possible to estimate any change in the water content of the plasma.

Table I shows the percentage recovery of the original amount of palmitic acid to be 99·5 %, of oleic acid 95·9 %, of a mixture of palmitic and oleic acids 98 %, and of a mixture of plasma extract and palmitic and oleic acids 106 %. The average deviation is $\pm$ 2·52 %, and all results are within 5 % of the theoretical values.

Error of method. In our estimations triplicate portions were used to determine the fatty acid content of the blood-plasma sample, and the best two of the three titrations were used for calculation. In this manner, the error of the method, taking the average of 266 triplicate determinations, was found

to be ± 11 mg./100 cc. This value is equal to a deviation of ± 1·6 % from the average for resting dogs (673 mg./100 cc.). For the maximum and minimum values the error is ± 1·1 % and ± 2·8 % respectively.

The average values varied with the species. Those found for man by Wilson and Gildea[1] (481 mg./100 cc.), using the present method are not so different from those of Nicholls and Perlzweig [1927–28] who determined the fat content of human blood using a modification of the method of Stewart and White [1925] and found it to be 426 mg./100 cc. Stoddard and Drury [1929], using their own method, also worked on human beings and reported an average fatty acid content of 294 mg./100 cc. in a series of 10 blood samples. Bloor *et al.* [1922] employed his nephelometric method and found an average value of 590 mg./100 cc. for dogs, while with his newer oxidation method [1928] he obtained slightly higher values. All these investigators used methods based on that of Kumagawa and Suto [1908] who employed a solvent for fatty acids to extract them from the blood, the subsequent steps, however, differing according to the various modifications.

The constancy of recovery obtained by our method has been shown above.

Results obtained by other methods yield the same general implications as to the deviations in blood-fat. The average deviation of a series of animals observed by Terroine [1914–15] at weekly intervals was ± 4·5 %, but the widest differences were ± 30 %. Bloor [1914] found the greatest variation of any animal to be ± 12 %, while the average was ± 6 %. Patterson [1927] noted that the fasting fat content for given individuals over a considerable period of time exhibited but slight variations, the widest being ± 6·5 % with an average deviation of ± 3 %.

Observations of a series of animals under postabsorptive conditions. In a large number (266) of observations on various animals, the minimum fat value for a normal resting dog was found to be 428 mg./100 cc., while the maximum was 906 mg./100 cc. This indicates a variation of 111 % for different individuals. Terroine's [1914–15] results were not unlike ours, as he found the extreme values to be 314 mg./100 cc. and 669 mg./100 cc. respectively, the variation being, therefore, 113 %. According to Bloor [1914], the average deviation of any animal in his series was ± 7 %, the greatest being ± 14 %, while Nicholls and Perlzweig [1927–28] give the blood values as varying between 333 and 492 mg./100 cc. in human subjects.

Summary.

A modification of the method of Stewart and White [1925] for the determination of blood-fat is presented. The experimental error of this method is ± 11 mg./100 cc., which represents a percentage deviation of ± 1·6, since 673 mg./100 cc. is the average value for dogs in the postabsorptive state. The level of blood-fat varied by ± 3·6 % in the course of an observation period

[1] Personal communication from Doctors E. F. Gildea and W. R. Wilson, who obtained six and three results respectively on normal human subjects.

of 8 hours, and therefore a difference of 40 mg./100 cc. has been considered significant. It was found that the values for blood-fat in postabsorptive conditions not only varied widely (111 %) in a given species (dog), but that the average values differed in various species. From an examination of the interfering substances it has been revealed that the method does not estimate the fatty acids which exist in the blood as soaps, but only those in ester combination.

We wish to thank Miss Katharine Humphreys for helping to revise the manuscript.

REFERENCES.

Bloor (1914). *J. Biol. Chem.* **19**, 130.
—— (1916, 1). *J. Biol. Chem.* **25**, 577.
—— (1916, 2). *J. Biol. Chem.* **26**, 417.
—— (1928). *J. Biol. Chem.* **77**, 53.
—— Pelkan and Allen (1922). *J. Biol. Chem.* **52**, 191.
Boyd (1931). *J. Biol. Chem.* **91**, 1.
Kumagawa and Suto (1908). *Biochem. Z.* **8**, 212.
Nicholls and Perlzweig (1927–28). *J. Clin. Invest.* **5**, 195.
Patterson (1927). *Biochem. J.* **21**, 958.
Stewart and White (1925). *Biochem. J.* **19**, 840.
Stoddard and Drury (1929). *J. Biol. Chem.* **84**, 741.
Terroine (1914–15). *J. Phys. Path.* **16**, 212.

CXCVIII. THE MICRO-DETERMINATION OF SODIUM IN BIOLOGICAL MATERIALS.

By ROBERT ALEXANDER McCANCE and HENRY LEIGH SHIPP.

From the Biochemical Laboratory, King's College Hospital, London, S.E. 5.

(*Received August 20th, 1931.*)

In a recent paper McCance and Shipp [1931] described a colorimetric method for determining very small amounts of sodium (0·02–0·8 mg.). The present paper describes the application of this method to the various body-fluids and solid tissues. By employing suitable protein precipitants the necessity for incineration has been avoided, except in the case of solid organs. This has great advantages: (1) in dealing with very small amounts of material; (2) there is always a risk of losing sodium by volatilisation; (3) there is a great saving of time; (4) no special apparatus is required.

(1) *Whole blood and serum.* Barrenscheen and Messiner [1927] described the determination of sodium in serum by means of a uranyl zinc acetate precipitation without incineration, but in our hands their technique has not proved satisfactory.

Grigaut and Boutroux [1930] have published a method based on the removal of the proteins by alcoholic uranium acetate. The sodium is then determined gravimetrically or volumetrically. Their results tend to be too high owing, they state, to the space occupied by the precipitated proteins. They do not state whether volume changes due to the addition of alcohol may not also contribute to the high results.

We have used trichloroacetic acid to precipitate the proteins. At the acid reaction the proteins are not precipitated as their sodium salts. The procedure is as follows. To about 8 cc. of water in a 25 cc. flask add 0·5 cc. of serum (or 1 cc. of whole blood). Add then with shaking 1 cc. of 30 % trichloroacetic acid (or 3 cc. in the case of whole blood). Allow to stand for 5 minutes, make up to the mark, mix and filter through an ash-free paper. It is important not to have too large a volume of liquid in the flask before adding the trichloroacetic acid. 1 cc. of the filtrate is then taken for each estimation and the sodium determined exactly as described in the previous paper. The sodium precipitate should be washed into 25 cc. flasks for colorimetric comparison. In Table I will be found a series of comparisons between this method of removing the blood- or serum-proteins and an incineration method. For the latter 1 cc. serum or 2 cc. whole blood were subjected to incineration in a silica crucible just below dull red heat, the product extracted 5 times with boiling *N*/2 HCl and the whole made up to 50 cc.

Barrenscheen and Messiner [1927] directed in their paper that the serum-proteins be removed by an alcoholic zinc acetate reagent which removes the phosphates at the same time. Though this eliminates one stage of the method, we have not found it satisfactory, since, after removal of the proteins with alcoholic zinc acetate, the precipitation of the sodium is very slow and erratic; and, in one or two cases, we have found that no sodium at all was precipitated in 1 hour. If left for a long time the precipitation of the sodium by the uranyl zinc acetate is probably complete, but the precipitate sticks to the sides of the tube, and centrifuging and washing are not so easy as they are after incinerating or employing trichloroacetic acid. Recoveries of sodium added to serum and blood by the trichloroacetic acid method will be found in Table II.

Since lithium interferes with the estimation of sodium as sodium uranyl zinc acetate [Caley and Foulk, 1929] lithium oxalate must not be used as an anticoagulant. We have used ammonium oxalate for our whole blood determinations.

(2) *Cerebrospinal fluid.* The technique is exactly the same as for blood-serum. Although normal cerebrospinal fluid contains practically no protein, we employ trichloroacetic acid and filter if there is visible opalescence. We give in Table II some recoveries of varying amounts of sodium added to cerebrospinal fluids.

(3) *Urine.* In urine there is usually enough sodium available to enable macro-methods to be used and Poulsson's [1928] modification of Barrenscheen and Messiner's method [1927] is probably quite satisfactory. For investigations on small experimental animals or even insects a micro-method is desirable. We have accordingly tested our procedure on urines and give in Table II some of our recoveries of known amounts of sodium added to urine. An accurate 1 in 25 or 50 dilution of the urine is prepared. 1–2 cc. of this diluted urine are then taken for each determination which is carried through as previously described.

Table I. *Estimation of sodium.*

Material	By incineration mg. per 100 cc.	By direct precipitation mg. per 100 cc.
Serum	348	346
,,	391	394
,,	330	340
,,	344	341
Whole blood	167	177
,,	212	208
Human milk	36·4	38·4
,,	42·5	42·7
,,	45·7	47·0
Cow's milk	89·3	89·3
,,	89·9	92·5

(4) *Milk.* As in the case of blood the successful determination of sodium without incineration depends upon the use of a suitable precipitant for the milk proteins. Bromine was suggested as a protein precipitant by Rideal and Stewart [1897] and was more thoroughly investigated by Allen and Searle

Table II. *Recovery of added sodium.*

Material and amount to which addition was made		Na added mg.	Na recovered mg.	Na recovered %
Serum	0·5 cc.	1·00	1·04	104·0
,,	0·5 ,,	2·00	2·11	105·5
,,	0·5 ,,	1·00	1·02	102·0
,,	0·5 ,,	2·00	1·96	98·0
,,	0·5 ,,	3·00	2·85	95·0
,,	0·5 ,,	1·20	1·17	97·5
,,	0·5 ,,	2·00	2·08	104·0
,,	1·0 ,,	4·00	4·20	105·0
Whole blood	1·0 ,,	1·00	0·96	96·0
C.S.F.	0·5 ,,	1·00	1·03	103·0
,,	0·5 ,,	1·00	0·99	99·0
,,	0·5 ,,	2·00	1·92	96·0
Urine	1·0 ,,	2·00	1·90	95·0
,,	1·0 ,,	2·00	2·04	102·0
,,	1·0 ,,	2·00	2·04	102·0
,,	1·0 ,,	2·00	1·95	97·5
,,	1·0 ,,	0·67	0·64	95·0
Milk (cow)	5·0 ,,	4·00	3·97	100·5
,, (human)	2·0 ,,	0·8	0·805	100·5
,, ,,	2·0 ,,	1·5	1·57	104·5
,, ,,	2·0 ,,	2·0	1·98	99·0
Fish	20 g.	10·0	10·0	100·0
,,	20 ,,	10·0	9·8	98·0
Meat	20 ,,	15·0	15·5	103·0
,,	20 ,,	15·0	15·5	103·0

[1897], but in recent years it has not received much attention. It is, however, an admirable reagent for the purpose in hand: (1) because it is easily prepared and kept sodium-free; (2) because it gives a beautiful protein precipitation; (3) because the proteins are not precipitated as the sodium salts. 5 cc. of milk are placed in a 50 cc. flask and diluted to about 15 or 20 cc. with distilled water. 10 cc. saturated bromine water (for human milk) or 15 cc. (for cow's milk) are then added, and after shaking the volume is made up to 50 cc. and the suspension filtered through an ash-free paper. Slight excess of bromine water does no harm and the filtrate should be yellow. 1–1·5 cc. of the filtrate are taken for each estimation, the phosphates removed with zinc acetate in 50 % alcohol and the sodium determined by precipitating it with uranyl zinc acetate in the manner already described. In Table I are shown the results obtained when this method was compared with an incineration method and in Table II the recoveries of known amounts of sodium added to milk.

(5) *Solid tissues.* Incineration is the standard method and has been practised in various forms by almost everyone who has studied this subject. It presents no difficulties provided a suitable temperature can be maintained. The uranyl zinc acetate method of determining sodium was shown in the previous paper [McCance and Shipp, 1931] to be satisfactory for hydrochloric acid extracts of incinerated material. There is therefore no need to discuss this point further here.

5–10 g. of finely minced tissue (1–2 g. of dried material) are placed in a

silica crucible, diam. 6·5 cm., height 4·5 cm. If not dry the tissue is then dried off on a sand bath. This process is accelerated by adding alcohol.

Although not essential an electric muffle furnace with graduated resistance is undoubtedly the most convenient method of effecting the incineration. We have, however, used the following apparatus quite successfully. A layer of iron filings 5 mm. deep is placed in an iron crucible 6·0 cm. high by 9·0 cm. wide. On this layer stands a nickel crucible 7·5 × 7·5 cm. fitted with a porcelain lid. Round the top of this crucible 4 holes are drilled 1 cm. in diameter. This allows access of air to the interior. The annular space between the iron and nickel crucibles is packed with iron filings 3 cm. deep and the filings are covered with a layer of asbestos fibre. The silica crucible which contains the material to be ashed stands inside the nickel crucible. The iron crucible is heated by a 1″ Fletcher burner. The whole is inexpensive and the only part requiring occasional renewal is the external iron crucible.

At the commencement heat is gently applied till all fuming has ceased. The nickel crucible is then covered and heating continued strongly for half an hour. The whole is then allowed to cool, the silica crucible is removed and placed on a large sheet of white paper and the charred mass broken up with a glass rod, any scattered fragments being replaced. The crucible is then replaced and heating continued with occasional stirring of the powder till the residue is white or almost so, otherwise the sodium is not extracted from the ash. The residue in the crucible is then repeatedly extracted with $N/2$ HCl and made up to 100 cc. after cooling: 1 or 2 cc. are taken for the sodium estimation.

Sodium added to tissues may be recovered quantitatively by this method (Table II). Each investigator must satisfy himself that the incineration temperature he employs will not cause volatilisation of the sodium.

Summary.

The technique of a micro-determination of sodium is described (*a*) without incineration in blood-serum, milk, cerebrospinal fluid and urine, (*b*) in solid tissues by incineration.

R. A. McCance has held a part-time and H. L. Shipp a whole-time grant from the Medical Research Council during the course of this investigation. We wish to take this opportunity of making acknowledgement.

REFERENCES.

Allen and Searle (1897). *Analyst*, **22**, 258.
Barrenscheen and Messiner (1927). *Biochem. Z.* **189**, 308.
Caley and Foulk (1929). *J. Amer. Chem. Soc.* **51**, 3233.
Grigaut and Boutroux (1930). *Compt. Rend. Soc. Biol.* **104**, 872.
McCance and Shipp (1931). *Biochem. J.* **25**, 449.
Poulsson (1928). *Biochem. Z.* **193**, 423.
Rideal and Stewart (1897). *Analyst*, **22**, 228.

OBITUARY NOTICE.

JAMES LORRAIN SMITH (1862–1931).

THE much regretted death, in April of this year, of Professor Lorrain Smith of Edinburgh calls for notice in this *Journal*, on account of his contributions on the chemical side to pathology and physiology. The son of a Free Church Minister in Dumfriesshire, he was born in 1862. He studied with great distinction at Edinburgh University, graduating first in Arts, and then in Medicine in 1889. He then went to study and work in Burdon Sanderson's Laboratory at Oxford, and soon afterwards began research work, along with Haldane, on the physiological action of air vitiated by respiration. This resulted in two well-known papers, followed by an independent one on the general metabolism after thyroidectomy. He was then appointed John Lucas Walker Student in Pathology at Cambridge, and later Demonstrator, under Roy, his time there being, however, interrupted by visits to von Recklinghausen's Laboratory at Strasburg to study histology, and to Bohr's Laboratory at Copenhagen to study methods of blood-gas analysis. In 1894 he was appointed Lecturer in Pathology at Queen's College, Belfast, and Professor in 1901.

It was here that he and Haldane carried out the experiments, first on man, and then on animals, from which they concluded that active secretion of oxygen inwards occurs in the lungs, though it was not known to them at the time that the secretion only occurs in response to the stimulus of want of oxygen in the tissues. They also worked out a method for determining in man the total volume and oxygen-capacity of the blood, this method depending on their discovery that the oxygen-capacity and colouring-power of the blood run parallel. With the new method Lorrain Smith investigated various abnormal conditions of the blood, and found that in the so-called anaemia of chlorosis the volume of the blood is in fact greatly increased, but correspondingly diluted as regards haemoglobin, so that the total haemoglobin in the body is unaltered. Other forms of anaemia were found to be true anaemias, with great diminution of the total haemoglobin; while cases with a high percentage of haemoglobin in the blood were found to have also a high blood-volume. Another of his discoveries about the same time was that oxygen at high partial pressures (even less than one atmosphere) produces pneumonia.

In 1904 he was appointed Professor of Pathology at Manchester and in 1912 to the Chair at Edinburgh. He had meanwhile, owing to his wide knowledge and sympathies, become more and more immersed in University and public affairs, leaving him less time for research. He became greatly interested, however, in the chemical conditions which determine the staining of acid or neutral fats, and the light thus thrown on physiological or pathological processes, and

he continued, along with his colleagues, to work at this subject till his death, making a number of discoveries, published in one paper after another.

In connection with the War he and colleagues in his laboratory introduced the well-known powerful antiseptic "eusol" (Edinburgh University solution) containing hypochlorous acid. They also did valuable work on "trench-foot," and he was probably the first to suggest the use of charcoal in respirators.

As a teacher Lorrain Smith was extremely successful, but perhaps even more so in his contributions to University policy and administration. He also served with distinction on various public bodies, and in 1906 he took a leading part in founding the Pathological Society. In 1909 he was elected a Fellow of the Royal Society, and he received honorary degrees from two universities.

J. S. H.

CXCIX. THE FORMATION OF BACTERIAL PROTEASES, ESPECIALLY IN SYNTHETIC MEDIA.

By RAYMOND BENNETT HAINES.

From the Low Temperature Research Station, Cambridge.

(*Received September 10th, 1931.*)

Brunton and Macfadyen [1889] showed that broth cultures of bacteria, moderately heated to kill the organisms, could bring about digestion of proteins. However, Fermi [1890] was apparently the first conclusively to demonstrate that an active proteolytic enzyme can be obtained from a sterile cell-free extract of the medium in which certain bacteria have been grown. Since that time difference of opinion has existed as to whether it is essential for the elaboration of such enzymes to have present in the medium proteins or protein derivatives. Some authors have reported the presence of enzymes after the growth of bacteria in synthetic media containing amino-acids only, or in some cases ammonium salts only, as the source of nitrogen. Others, on the other hand, have maintained that "organic" nitrogen in the form of peptone or some other complex substance, is required for protease formation. Much of the earlier work seems unreliable when viewed from a modern standpoint, since

(*a*) the concept of p_H, its application to bacteriology and its fundamental importance in enzymic activity had not been elaborated;

(*b*) the significance of vitamins in nutrition was unknown and discordant results with synthetic media may have been due to their presence in the amino-acids or other substances used;

(*c*) the organisms were not washed and consequently organic matter was probably carried over from the stock culture to the synthetic medium.

Bainbridge [1911] showed that pure native proteins are not attacked by bacteria and his results were confirmed and enlarged by Sperry and Rettger [1915]. Diehl [1919] stated that amino-acids are required in the medium for the elaboration of enzymes and he reported a curious specificity according to the amino-acid present. Merrill and Clark [1928], however, found that they obtained gelatinase from organisms growing in a synthetic medium of carefully purified salts, with ammonium chloride as the source of nitrogen, provided that salts of calcium and magnesium were present. No gelatinase was obtained in the absence of these salts, despite good growth. Wilson [1930] could not confirm Diehl's specificity. She criticised Merrill and Clark's results on the ground that poor growth only was obtained in the absence of salts of calcium

and magnesium and concluded that these salts are necessary for the elaboration of enzymes only in so far as they stimulate growth.

Numerous points remain to be settled. It is not evident from Bainbridge's work whether failure of the organism to grow was due to inability to utilise pure protein as a source of nitrogen or to lack of some other essential constituent such as the salts mentioned above. Non-decomposition of the protein may have been due as much to the inability of the enzyme to attack the protein, owing to high dilution or other non-optimal conditions, as to failure of the organism to produce enzyme. It is essential to guard against misinterpreting effects which may be due merely to dilution. For example, in the course of the present work it has been found that by diluting an enzyme preparation which formerly attacked two proteins, it may be made to attack only the one. If this latter dilution had been arbitrarily selected in the first instance a case of specificity might have been assumed. Again, if the proteolytic enzymes are concerned in the synthetic processes of the cell as well as in the degradation processes, it is to be expected that these enzymes will be formed whenever good growth occurs. In view of the conflicting views advanced, this latter point requires further investigation, and it is the main purpose of the present paper to attempt to settle it. It was necessary first of all to make a preliminary study of the formation of proteases in broth culture by the organisms selected (*i.e.* under optimum conditions) by way of control. The work was then extended, as far as possible with the same organisms, to synthetic media of known composition.

Protease formation in broth.

Methods.

A single loopful of an overnight culture of the organism to be studied was inoculated into several hundred cc. of nutrient broth, p_H 7·4, spread in a thin layer in a large Fernbach flask. After incubation, during which it had been repeatedly shaken to ensure aeration of the culture, the broth was centrifuged at high speed for 30 minutes and sterilised by filtration through a Chamberland candle. As the result of preliminary tests 2–5 cc. lots of the filtrate were transferred to tubes containing 5–10 cc. of gelatin, caseinogen or egg-albumin solution. These were then incubated for several days and digestion estimated by the methods outlined below. The gelatin was made up according to the method of Wilson [1930] by dissolving 5 g. gold label gelatin in 100 cc. phosphate buffer on a water-bath at 60°, adding phenol to 0·5 % and adjusting the final p_H at 7·4. An approximately 1 % caseinogen solution was prepared by well stirring the required amount with phosphate buffer containing 0·9 % sodium chloride, filtering and adding thymol solution to 1 %, the final p_H again being 7·4. Egg-albumin was obtained from fresh eggs by the modified method of Hopkins used by Adair and Robinson [1930] and twice crystallised. It was dialysed in collodion sacs until a negative test for sulphates was obtained in the liquid outside the sac. Liquefaction of the gelatin was tested by immersing

the tubes direct from the incubator in ice-water, and comparing the time required for setting with that of a control tube. Caseinogen digestion was indicated by absence of precipitate or turbidity on adding 0·4 cc. of alcoholic acetic acid (Diehl). Controls were here again carried out. Digestion of egg-albumin was estimated by adjusting the p_H of the solution approximately to the isoelectric point, coagulating by immersing for five minutes in boiling water, filtering either through Jena sintered glass filters or washed and tared filter-papers, drying at 100° for 4 hours and weighing.

Results.

Table I is an example of the type of result obtained with broth cultures of the organisms listed.

Table I.

	Gelatin		Caseinogen		Egg-albumin	
Organism	Blank	Exp.	Blank	Exp.	Blank g.	Exp. g.
Proteus 3	set 3 mins.	*not* set 24 hrs.	0	+ +	0·100	0·097
B. mesentericus	,, 3 ,,	,, ,,	0	+ +	0·098	0·084
Pseudomonas 1	,, 4 ,,	,, ,,	0	+ +	0·098	0·100
Pseudomonas 2	,, 3 ,,	,, ,,	0	+ +	0·088	0·077

In the first gelatin column is given the time of setting of the same mixture of enzyme and gelatin as in the second column, but heated to 100° for 10 min. In the egg-albumin column the mean weights of the respective coagula are given. In this and following tables these conventions are used for digestion of caseinogen: 0 = no difference from blank; * = doubtful; + = partial digestion; + + = complete digestion.

The organism *Proteus 3* was a stock culture from the London School of Hygiene[1] and thought to be Felix and Weil's *x* 19 strain. *Bacillus mesentericus* was a stock laboratory strain from the Pathological Laboratory, Cambridge[2]. *Pseudomonas 1* was isolated by the author from an abattoir but had been kept in culture for some months, and *Pseudomonas 2* was freshly isolated from cow-dung.

It will be seen that although sterile filtrates from all the above organisms liquefied gelatin and digested caseinogen beyond the acid-coagulable stage, filtrates from *Proteus 3* and *Pseudomonas 1* were without action on the native egg-albumin. Slight digestion beyond the heat-coagulable stage was apparently obtained with *B. mesentericus* and *Pseudomonas 2*. The latter organism was freshly isolated and sown immediately into broth, because it was thought that non-digestion of the albumin might be due to the fact that the stock strains had lost much of their vigour on repeated subculture. That this was not the case is evident from Table II in which it is seen that *Pseudomonas 2* produced a gelatinase so active that 0·01 cc. of filtrate liquefied 5 cc. of gelatin, while equal volumes of filtrate and albumin solution resulted in only the slight decomposition of albumin shown in Table I. Gelatin is apparently more easily attacked than caseinogen, since the higher dilutions which still liquefied gelatin were without action on the caseinogen.

[1] Supplied by courtesy of Dr Anderson.

[2] Supplied by courtesy of Dr Graham-Smith.

Table II. *Effect of dilution.*

Pseudomonas 2 grown for 8 days at 20°, filtrate incubated for 3 days at 37°.

Volume of filtrate added to 5 cc. gelatin or caseinogen cc.	Gelatin		Caseinogen	
	Blank	Exp.	Blank	Exp.
0·01	set 3 mins.	*not* set 24 hrs.	0	0
0·05	,, ,,	,, ,,	0	*
0·10	,, ,,	,, ,,	0	+
1·00	,, ,,	,, ,,	0	+ +
2·00	,, ,,	,, ,,	0	+ +

Apparently, therefore, little or no decomposition of pure native albumin beyond the heat-coagulable stage occurred after incubation with filtrates containing a very active gelatinase and a moderately active casease. Failure to decompose the albumin can hardly in this instance be explained on the basis of specificity of amino-acids in the food supply, for there are presumably present in meat-extract peptone broth all the amino-acids required. Several possibilities present themselves, but further experimental evidence with better methods is desirable. Since the main object of this paper was the study of protease formation in synthetic media, the point has not at present been pursued further.

Protease formation in synthetic media.

Methods.

In the main Merrill and Clark's simple medium [1928], the media of Diehl [1919] and Fränkel [1894], or slight modifications of them, have been used. The exact composition of these media as used is listed in the Appendix. Pyrex glassware was used throughout. It was cleaned carefully by standing for 24 hours under strong chromic acid, 24 hours under strong alkali, several days under distilled water repeatedly changed and was finally autoclaved at two atmospheres pressure for one hour. Ammonia-free redistilled water was used exclusively. All salts, being of A.R. standard to begin with, were recrystallised two or three times. Glycerol and lactic acid were distilled once *in vacuo*. Glucose was crystallised three times from a mixture of alcohol and water. Good samples of crystalline asparagine and glycine were recrystallised from water. In the case of Merrill and Clark's and Fränkel's media, stock solutions of each of the constituents were made up separately in sterile water and finally sterilised by steaming once for 15 minutes. The sterilisation of the mixture in the final medium, which invariably resulted in decomposition of the glucose, was thus avoided, the mixture being made up by dilution from stock solutions with aseptic precautions. In all cases the final p_H of the medium was adjusted prior to inoculation to 7·4 approximately by the colorimetric method, using $N/5$ NaOH stored in a waxed bottle fitted with delivery burette. The organisms were grown on the surface of agar plates at the appropriate temperature overnight. The surface growth was then carefully scraped off with a sterile platinum needle, taking care not to break into the agar, and suspended in saline. After

centrifuging at high speed for 30 minutes the deposit was ground up in a fresh quantity of sterile saline and the centrifuging repeated. In all, at least 3 washings were carried out. 1–2 cc. of the final suspension of about 50 cc. were inoculated into the various media. From 20 to 50 cc. of culture fluid were used in a thin layer on the bottom of 250–500 cc. Erlenmeyer flasks in order to ensure adequate aeration. Incubation was in general carried on from 2–5 days, for although the final growth was in many cases as luxuriant as in broth culture, the rate of growth was noticeably slower at the beginning. Doubtful cases were incubated for a week or more. At least ten subcultures were made to show that the organism was capable of continued growth in the synthetic medium. As a rule those organisms which could not flourish in the medium chosen died out at about the third subculture. Protease formation was measured in a similar manner to that used for broth cultures. Egg-albumin was dispensed with since the broth controls of the organism had given so little breakdown.

Results of experiments on growth in synthetic media.

The results of the experiments to determine the viability of the organisms in the respective media are summarised in Table III. *Proteus 1* was a strain of *P. vulgaris* obtained from the National Collection of Type Cultures, *Proteus 2* was isolated by the author from putrid ox-liver, *Proteus 3* was obtained as mentioned previously from the London School of Hygiene and was probably Felix and Weil's *x* 19 strain. "*Proteus 4*" was stated by one authority to be a true *Proteus*, by another to be a *Zopfius*.

It will be seen that the *Proteus* strains 1, 2 and 3 all consistently failed to grow in any of the synthetic media used. Slightly better growth was obtained in a medium containing the following unpurified amino-acids: glycine, alanine, asparagine, tryptophan, histidine, cysteine, cystine and tyrosine. Even here, however, growth failed after 4–5 subcultures. The passage of oxygen through the culture also had a stimulating influence but failed to maintain continued growth. "*Proteus 4*" (*Zopfius* ?) on the other hand grew well in Clark's and Diehl's media, provided salts of magnesium were present. In this connection it is interesting to note that Merrill and Clark [1928] reported good growth of "Proteus bacteria" in their synthetic media, whilst Wilson [1930] and Koser and Rettger [1919] obtained negative results in a variety of media. It is possible, but not yet proven, that certain strains of true *Proteus* organisms belong to the group which Hosoya and Kuroya [1923] showed would not grow without the presence of vitamin B. A broth control experiment indicated that protease formation with "*Proteus 4*" was feeble. Attempts to work with *Proteus* organisms had therefore to be abandoned. *B. mesentericus* and organisms of the *Pseudomonas* group proved suitable. Luxuriant growth was obtained in Merrill and Clark's simple medium with these bacteria provided salts of calcium and magnesium were present. Whilst requiring about 3 days to reach maximum turbidity as compared with 24 hours in broth, the final growth as

judged by turbidity was equally good in both cases. Growth in the absence of both calcium and magnesium was either feeble or definitely negative. In the presence of calcium but the absence of magnesium growth was little if any better than in the absence of calcium. In the presence of magnesium, on the other hand, luxuriant growth was again obtained. Good growth was also obtained in Diehl's medium which contained magnesium but no calcium. Apparently salts of magnesium are essential for the continued growth of bacteria in these simple synthetic media with an inorganic source of nitrogen. Such a salt is not universally necessary for growth, because continued multiplication of the bacteria took place in Fränkel's medium which contained no salt of calcium or magnesium. This medium, however, contains both ammonium lactate and asparagine. Even here these salts exerted a stimulating influence on growth, because, although good growth was obtained in their absence, more rapid and somewhat more luxuriant growth took place in their presence.

Protease formation.

The results of a number of experiments are summarised in Table IV. It will be seen that good protease formation took place in Merrill and Clark's medium in the presence of salts of calcium and magnesium, gelatin being liquefied and caseinogen digested. Little or no protease could be detected in the presence of calcium without magnesium in this medium, the growth, however, being relatively poor. Nevertheless counts showed that fair multiplication had occurred. In the same medium together with magnesium salts only luxuriant growth occurred in every way as good as in the presence of both salts. Yet in the latter case protease formation was negative or at the best very doubtful. Hence the position taken up by Wilson [1930] that these salts influence protease formation only in so far as they stimulate growth is not tenable. Either calcium salts alone, or a mixture of calcium and magnesium salts, have a specific action on the liberation of proteases in this medium. Magnesium salts alone have not this ability, or possess it only to a very slight extent, although they stimulate growth. The action of calcium salts only, or a mixture of calcium and magnesium salts, apart from their growth-stimulating properties, could not be tested in Merrill and Clark's medium, because so little growth occurred in their absence. A medium was required which would give good growth in the absence of these salts as well as in their presence. After some preliminary tests a slight modification of Fränkel's medium [1894] was adopted, as mentioned above. Continued growth of both *Pseudomonas* and *B. mesentericus* was obtained in this solution. All attempts to demonstrate the presence of protease in the medium without any addition of salts of calcium and magnesium have consistently failed, despite good growth with both organisms. A little enzyme was detectable in the presence of calcium salts, but good protease formation occurred on the addition of both calcium and magnesium salts. That the action of these salts was not due to activation of the enzyme during the incubation of a filtrate containing them and protein was shown by the fact that no difference

Table IV.

Temperature of incubation 37°.

Organism	Medium	Growth	Temp. of incubation of culture ° C.	No. of sub-culture	Age of sub-culture days	Time of incubation of filtrate hrs.	Protease formation: Gelatin: Blank	Protease formation: Gelatin: Exp.	Protease formation: Caseinogen
Pseudomonas 2	Clark with Ca and Mg salts	+ + +	20	3	8	48	Set 3 mins.	>10 hrs.	+ +
,,	Clark without Ca and Mg salts	+	20	3	8	48	,, 3 ,,	3 mins.	0
,,	Clark with Ca salts only	+ +	20	3	7	48	,, 3 ,,	7 mins.	*
,,	,,	+ +	20	4	8	48	,, 3 ,,	6 mins.	*
,,	,,	+ +	20	5	4	48	,, 3 ,,	15 mins.	*
,,	Clark with Mg salts only	+ + +	20	3	7	48	,, 4 ,,	4 mins.	*
,,	,,	+ + +	20	4	8	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	5	4	48	,, 3 ,,	4 mins.	0
,,	Diehl	+ +	20	4	7	48	,, 4 ,,	5 mins.	0
,,	Fränkel alone	+ + +	20	5	9	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	8	4	48	,, 3 ,,	3 mins.	0
,,	Fränkel with Ca and Mg salts	+ + +	20	2	2	48	,, 3 ,,	>10 hrs.	+ +
,,	,,	+ + +	20	3	5	48	,, 3 ,,	>10 hrs.	+ +
,,	,,	+ + +	20	5	4	48	,, 4 ,,	>10 hrs.	+ +
,,	Fränkel with Ca salts only	+ + +	20	2	2	48	,, 3 ,,	3 mins.	*
,,	,,	+ + +	20	5	5	48	,, 3 ,,	4 mins.	0
,,	Fränkel with Mg salts only	+ + +	20	2	2	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	5	4	48	,, 3 ,,	4 mins.	*
B. mesentericus	Clark with Ca and Mg salts	+ + +	37	1	5	48	,, 4 ,,	>10 hrs.	+
,,	,,	+ + +	37	2	6	48	,, 4 ,,	>10 hrs.	+
,,	,,	+ + +	30	8	11	48	,, 3 ,,	>10 hrs.	+ +
,,	Clark with Ca salts only	+ +	30	3	11	48	,, 3 ,,	3 mins.	*
,,	Clark with Mg salts only	+ + +	30	3	11	48	,, 3 ,,	5 mins.	0
,,	,,	+ + +	37	1	5	48	,, 3 ,,	3 mins.	0
,,	Fränkel alone	+ + +	30	6	11	48	,, 3 ,,	3 mins.	*
,,	,,	+ + +	37	1	5	48	,, 3 ,,	4 mins.	0
,,	Fränkel with Ca and Mg salts	+ + +	37	3	6	48	,, 3 ,,	>10 hrs.	+
,,	Fränkel with Mg only	+ + +	37	3	6	48	,, 3 ,,	3 mins.	0

occurred on adding 0·2 cc. of their solutions to inactive filtrates together with protein, prior to incubation.

The original observation of Merrill and Clark is therefore confirmed. Salts of calcium and magnesium are apparently of fundamental importance to the bacterial cell for the liberation of proteases into the surrounding medium, in the media so far tested. The best effect is obtained by the combination of the two salts: magnesium salts alone have a stimulating influence on growth but affect protease production but little if at all. Calcium salts alone have no apparent stimulating influence on growth, but a trace of protease seems to be detectable in their presence. The action of these salts cannot be explained as a process of stimulation of growth only. So far, only ammonium chloride, ammonium phosphate, ammonium lactate and asparagine have been used as possible sources of nitrogen for the organism. All these can readily be obtained in a state of purity. It remains to be seen whether the action of these salts is of a fundamental nature and common to all media containing other and more complex sources of nitrogen. While there is as yet no experimental evidence, it is interesting to speculate that the action of salts of calcium and magnesium may in some way be linked up with their well-known characteristic effects at certain interfaces and their influence on the reversal of certain emulsions. It is possible that proteolytic enzymes are already present in the cell and assisting in the processes of metabolism, but that they are only secreted into the surrounding medium with a change in the cell surface caused by magnesium and calcium ions. In that case the calcium salts of serum may play an important rôle in the phenomena of immunity.

Summary.

1. Pure native egg-albumin is apparently more resistant to bacterial enzymes than commercial caseinogen or gelatin. Sterile filtrates of broth cultures of *B. mesentericus* and a *Pseudomonas* which readily attacked the latter had but little action on the albumin.

2. Mixtures of salts of calcium and magnesium, or magnesium salts alone, exert a stimulating influence on the growth of micro-organisms in simple synthetic media. Calcium salts alone have but little action in this respect.

3. Proteases are formed in simple synthetic media with ammonium chloride as the source of nitrogen provided that salts of calcium and magnesium are present. Little or no protease formation occurs in the presence of magnesium salts alone, despite good growth. In a medium containing ammonium lactate and asparagine protease formation is stimulated by these salts.

4. The influence of salts of calcium and magnesium on protease formation cannot be explained on the basis of stimulation of growth only.

The author wishes to express his thanks to Sir William B. Hardy who suggested the problem, to Dr G. S. Graham-Smith for advice and the supply of

cultures, to Dr C. G. Anderson for the supply of cultures, and to Mr J. Barlow for assistance in the experimental work.

REFERENCES.

Adair and Robinson (1930). *Biochem. J.* **24**, 993.
Bainbridge (1911). *J. Hyg.* **11**, 341.
Brunton and Macfadyen (1889). *Proc. Roy. Soc. Lond.* **46**, 542.
Diehl (1919). *J. Infect. Dis.* **24**, 347.
Fermi (1890). *Arch. Hyg.* **10**, 1.
Fränkel (1894). *Hyg. Rundschau.* **17**, 769.
Hosoya and Kuroya (1923). *Sci. Rep. Govt. Inst. Infect. Dis.* Tokyo, **2**, 233, 287.
Koser and Rettger (1919). *J. Infect. Dis.* **24**, 301.
Merrill and Clark (1928). *J. Bact.* **15**, 267.
Sperry and Rettger (1915). *J. Biol. Chem.* **20**, 445.
Wilson (1930). *J. Bact.* **20**, 41.

APPENDIX. *Composition of media used.*

Merrill and Clark's medium.

NH_4Cl	10 g.
$Na_2HPO_4, 2H_2O$	10 g.
KH_2PO_4	2 g.
Glucose	10 g.
Water	1 litre

5 cc. of 0·1 % $MgSO_4, 7H_2O$ and 2 cc. well shaken suspension of 0·1 % $CaCO_3$ in 50 cc. of medium where required. Final p_H 7·4.

Diehl's medium.

$(NH_4)_2HPO_4$	1·75 g.	
K_2HPO_4	0·25 g.	
$Na_2HPO_4, 2H_2O$	0·5 g.	Final p_H adjusted to 7·4
$MgSO_4, 7H_2O$	0·5 g.	
Glycerol	20·0 cc.	
Water	1 litre	

Fränkel's medium.

NaCl	5·0 g.	
KH_2PO_4	2·0 g.	Final p_H adjusted to 7·4
Ammonium lactate ...	6·0 g.	
Asparagine	4·0 g.	
Water	1 litre	

Calcium and magnesium salts added as above when required.

CC. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRECIPITATING AND PROTECTIVE ACTIONS OF MIXTURES OF TWO PROTEINS ON COLLOIDAL GOLD AND GUM BENZOIN.

By REGINALD HAYDN HOPKINS, WILLIAM OGILVY KERMACK and WALTER THOMAS SPRAGG.

From the Biochemical Laboratory, Heriot-Watt College, and the Research Laboratory of the Royal College of Physicians, Edinburgh.

(*Received September 23rd, 1931.*)

In a recent communication [Hopkins, Kermack and Spragg, 1931] the results were given of a series of experiments on the precipitation of colloidal gold and gum benzoin by the proteins, gelatin, ovalbumin, serum-albumin, the serum-globulins and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. Observations were also made on the protective actions of these proteins against precipitation of the colloids by 1·25 % sodium chloride. Except in the case of oxyhaemoglobin, the protective power was minimal in the neighbourhood of the isoelectric point of the protein. It seemed desirable to extend the observations to mixtures of two proteins in order to find out, for example, what occurs when conditions are such that one protein alone will precipitate and the other protect. It was decided for this purpose to limit the experiments to the three proteins, gelatin, ovalbumin and oxyhaemoglobin. Of these three, the first two have approximately the same isoelectric point, p_H 4·7, whilst that of the third is considerably higher, p_H 6·8. Further, gelatin has considerable protective power even in the neighbourhood of the isoelectric point, but ovalbumin has not, whilst the behaviour of oxyhaemoglobin is anomalous in that the minimum of protective action is at a p_H somewhat below that of the isoelectric point. These three proteins have been employed in pairs, and in each case two sets of observations have been made, colloidal gum benzoin being employed as lyophobic colloid in the one case and colloidal gold in the other. As in the previous experiments, it was desired to make readings over a range of p_H values. With each pair of proteins, sets of observations were made in which the p_H was kept constant but the concentration of each protein independently varied. Similar sets of observations were made at other p_H values. With each pair of proteins, either two or three sets of readings were made, each at a constant p_H. The values of p_H employed in the various cases were chosen so as to be likely to yield the maximum amount of information.

In all cases readings were made before and after the addition of sodium chloride.

Although mixtures of proteins are frequently encountered in practice, there appears to be comparatively little recorded in the literature in respect of the precipitating and protective actions of such mixtures. Reference may be made to a paper by Prideaux and Howitt [1931] on the rate of electrophoresis of gold sol in the presence of ovalbumin, gelatin and mixtures of the two. On the acid side of the isoelectric point, the rate of electrophoresis in the presence of the two proteins was considerably less than in the presence of either separately, whilst on the alkaline side, the rate was approximately the same as that observed in the presence of gelatin alone.

Experimental.

The general method of carrying out the experiments was analogous to that employed in the previous work [1931]. To each of a series of tubes were added 0·25 cc. of protein A and 0·25 cc. of protein B, each of suitable concentration, 0·25 cc. of a buffer solution and 1·0 cc. of the lyophobic colloid. The buffer solution contained $N/50$ phosphate and $N/50$ acetate. In any particular experiment the tubes were arranged in the form of a rectangular array. The tubes in any one row contained the same concentration of protein A but various concentrations of protein B, whilst the tubes in any one column contained the same concentration of protein B but various concentrations of protein A. The same buffer mixture was added to all the tubes in any one array, so that the final contents of all the tubes were approximately at the same p_{H}. This was ensured by previously adjusting the p_{H} of the protein solutions distributed to the tubes to be approximately that of the buffer mixture employed. To each tube of the array was added 1·0 cc. of either colloidal gum benzoin or of colloidal gold. The tubes were shaken and readings were made after 24 hours. 0·25 cc. of 10 % sodium chloride was then added to each tube, the tubes shaken again and read after a further 24 hours. The p_{H} of a number of tubes was then determined in order to ascertain the exact p_{H}, which, although approximately constant throughout the array was not necessarily the same as that of the buffer used, as the latter may be modified by the buffering substances present in the gum benzoin and the gold. As mentioned in the previous paper, the readings in the case of tubes containing appreciable quantities of oxyhaemoglobin could best be made by examining the contents in a beam from an arc lamp, when the presence of particles of gold throughout the solution could readily be detected by means of the Tyndall effect. In recording the results obtained with the gelatin-ovalbumin mixtures and gold sol, two observations were made for each tube, one referring to the colour and the other to the amount of gold which had settled to the bottom of the tube. In the case of experiments with gold in which oxyhaemoglobin was present, analogous observations of colour could only be made when the concentration of oxyhaemoglobin was small. In the previous paper these two methods of reading were not

clearly distinguished. When gold is precipitated by an inorganic salt the colour is changed from red to blue and precipitation follows. This precipitate is always quite blue. In the previous paper it was noted that in the presence of protein the precipitate was frequently red or purple, although all the gold had been deposited. The diagrams given showed the regions in which complete or almost complete colour change, or complete or almost complete precipitation, had been observed. It has become clear during the present work that it is desirable to distinguish between these two phenomena. As a consequence readings have been made in the case of gold, firstly on the basis of colour change alone, and secondly on the basis of degree of precipitation alone. The diagrams which are given have been prepared from the latter readings. It has been found that the two sets of readings did in fact follow each other fairly closely with regard to the general shape of the zones, but not with regard to the exact position of the zones. It also appears from the present results that the precipitates marked red or purple (by shading) in the previous paper, although distinctly reddish as compared with the normal blue precipitate of gold, were nevertheless considerably bluer than the unprecipitated gold. It follows from these considerations that the exact limits of precipitation of gold recorded in the new diagrams are not always completely in agreement with those given in the previous paper. This refers more particularly to the case of gelatin and gold. Fig. 8 shows the results when the new method of recording is employed. (Contrast with Fig. 1 A in the previous paper.) It will be noted that on the acid side of the isoelectric point, protection now occurs in the presence of lower concentrations of gelatin. This is because a tube showing an almost complete colour change is now in the non-precipitated region, as under these conditions with very low protein concentration, precipitation does not occur in general until the colour change is complete.

The experimental results are shown in Figs. 1 A to 8. The thick lines, whether continuous or dotted, represent approximately the concentrations at which partial precipitation occurs, so that they separate zones of precipitation from those of non-precipitation. The shading indicates that side of the line on which is the zone of precipitation. Each line is distinguished by a number, *e.g.* 5·6, which denotes the p_H value to which the line refers. The significance of dotted and continuous lines, thick and thin lines, will be explained in the discussion.

Discussion.

According to the theory of colloids which has been elaborated in recent years by Kruyt and others, the stability depends on two factors, (1) the existence of an electric charge on the particles, and (2) the existence of groups which have an affinity for the medium employed for the continuous phase. In the case of a lyophobic colloid protected by a lyophilic colloid such as protein, the effects of the protein may be exerted in two different ways, (1) by modifying the electric charge on the particles, and (2) by forming a film round the

particles, so modifying the surface forces that the particles will not adhere together even though electrically neutral. In this paper, we propose to refer to the former as the "electrostatic effect" and to the latter as the "proper protective effect." For example, the "proper protective effect" of ovalbumin is relatively small, and that of gelatin relatively great. It has been shown in the previous paper [Hopkins, Kermack and Spragg, 1931] that when a single protein is present these two factors explain completely the behaviour of certain lyophobic colloids in the presence of proteins, although, with other lyophobic colloids such as gold, the behaviour is less simple. The results of the present work indicate that this conclusion, so far as concerns colloidal gum benzoin, may be extended to the case where two proteins are present simultaneously. It is entirely in harmony with the above principles that the proper protective effect should be additive and should always exert itself whatever the electrostatic condition of the particles may be. The electrostatic effect is also additive in itself, but is subordinate to the combined proper protective effect.

Precipitation of colloidal gum benzoin. We shall first consider the pair of proteins, ovalbumin and gelatin. In the previous paper, dealing with single proteins, it was found convenient to represent the results diagrammatically in the form of graphs, one ordinate representing concentration of protein, the other p_H. The regions over which complete or almost complete precipitation occurred were then marked out. As, in the present instance, there are two proteins, a three-dimensional model is required to give a similar representation. It was found possible from the observations to construct a model of this kind showing the regions precipitated, the three variables being concentration of gelatin, concentration of ovalbumin and p_H. It should be added that, in constructing this model, considerable help was gained by taking into account the degree of precipitation in tubes which were not exactly at the boundary line between precipitation and non-precipitation. It is difficult to give an adequate

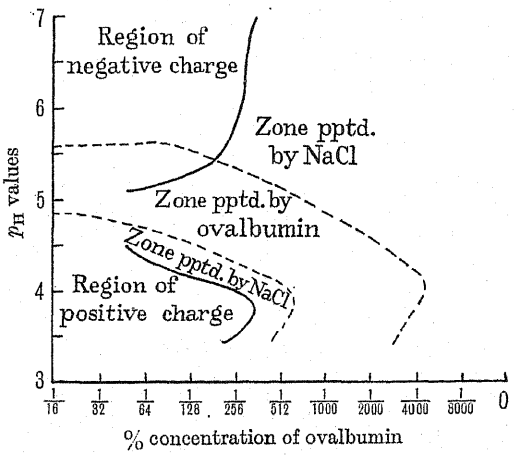


Fig 1 A. Action of ovalbumin on gum benzoin. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4·6–4·8.

representation of a model of this kind by two-dimensional diagrams, but some indication of its general nature may possibly be afforded by the "contour" diagrams given. Considering for the moment Figs. 1 A and 1 B, these refer to

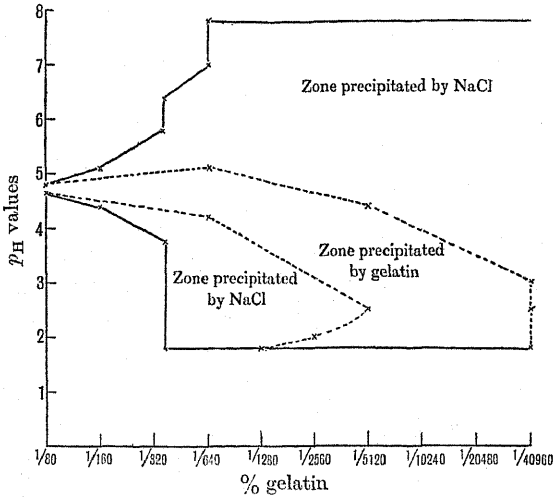


Fig. 1 B. Action of gelatin on gum benzoin. No buffers used.

ovalbumin and gelatin respectively alone, and so give a picture of the two adjacent faces of the model, which meet in an edge representing the p_H axis corresponding to zero concentration of both proteins. The region of precipitation in the three-dimensional model was in the form of a zone separating a region in which the particles possessed a positive charge from one in which they possessed a negative charge. This zone naturally had an under surface bounding the region of positive charge and an upper surface bounding the region of negative charge. The lines shown in Fig. 2 A may be regarded as contours drawn on these surfaces, each at a constant p_H. The parts of the contours which refer to the upper surface are represented by continuous lines and those referring to the lower surface by dotted lines. The thick continuous and dotted lines were obtained directly from the observations as mentioned in the experimental section. The thin continuous and dotted lines have been interpolated with the help of the model, and serve to indicate more fully the nature of the two surfaces, upper and lower. As mentioned in the experimental section, shading indicates on which side of the contours precipitation occurred.

The zone of precipitation comprises the region in which the particles would be expected to have a very low or zero charge, but it does not extend to any concentration of gelatin above a certain critical concentration. At this concentration the proper protective effect begins to function, so that, even at the isoelectric point, precipitation does not occur. Although the proper protective effect of the ovalbumin is not sufficient to prevent agglutination of the particles when their charge is zero, nevertheless it is not altogether non-existent. This is shown by the fact that, in presence of large concentrations of ovalbumin the

concentration of gelatin required for complete protection is not quite so great as when ovalbumin is absent (see Fig. 2 A).

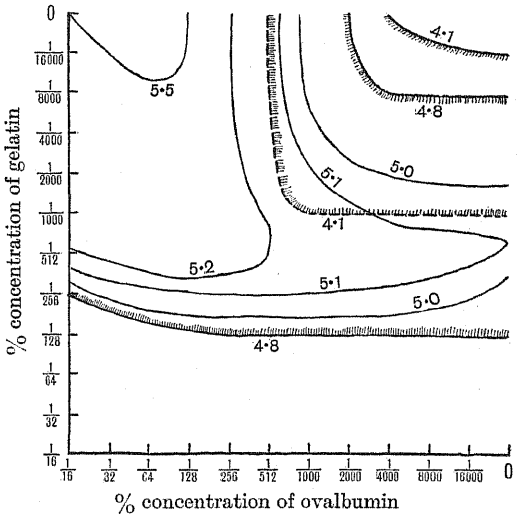


Fig. 2 A. Action of mixtures of gelatin and ovalbumin on gum benzoin at various [H·].

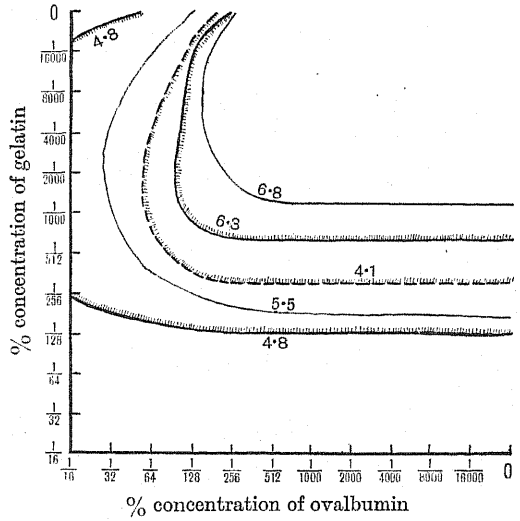


Fig. 2 B. Protective action of mixtures of gelatin and ovalbumin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

In the case of mixtures of gelatin and oxyhaemoglobin, the same general result is obtained, and in the three-dimensional model the chief difference as compared with the gelatin-ovalbumin system is that the zone of precipitation is moved to a region of higher p_H values in that part of the system which refers to low gelatin and high oxyhaemoglobin concentrations (see Fig. 3 A).

In the case of ovalbumin-oxyhaemoglobin mixtures, the zone of precipitation is extended in the direction of higher ovalbumin concentration in consequence of the low proper protective effect of this protein. Since the proper

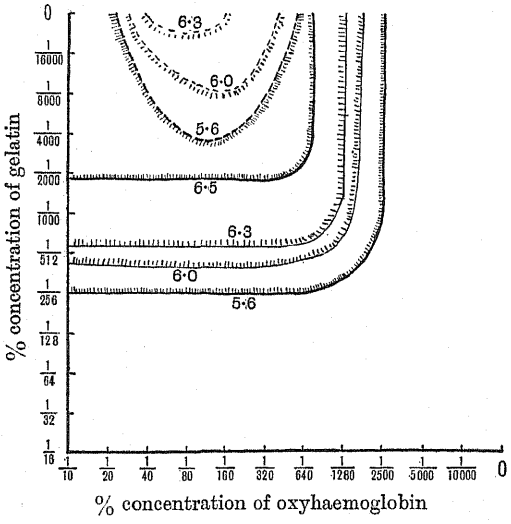


Fig. 3A. Action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various [H·].

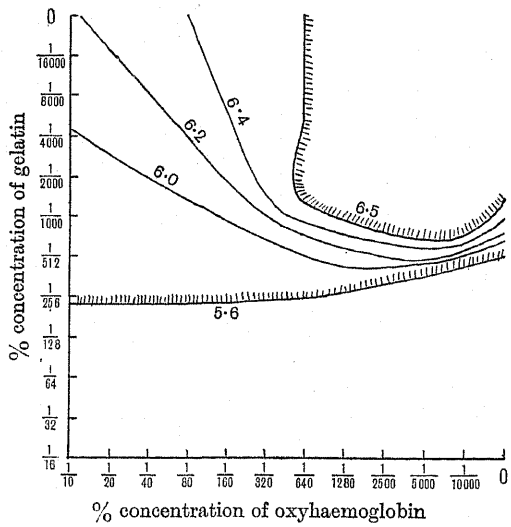


Fig. 3B. Protective action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

protective effects of these two proteins are small, the stability is almost entirely controlled by the electrostatic effects (see Fig. 4A).

Protection of colloidal gum benzoin. The results observed in the presence of sodium chloride may be discussed in the light of the same principles. The chief

function of salt is to reduce the charge on the particles which have no film of protein round them, or which have adsorbed only small quantities of protein.

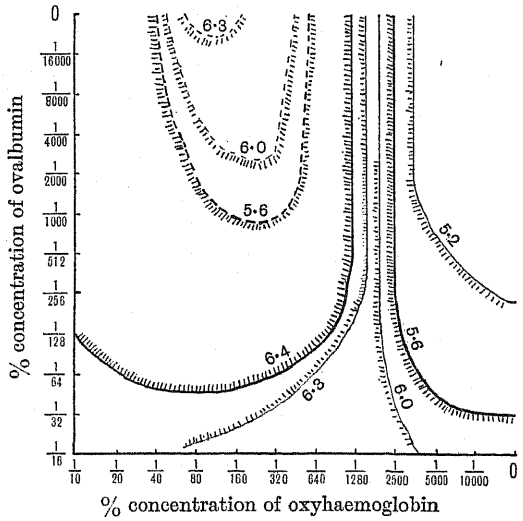


Fig. 4A. Action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various [H·].

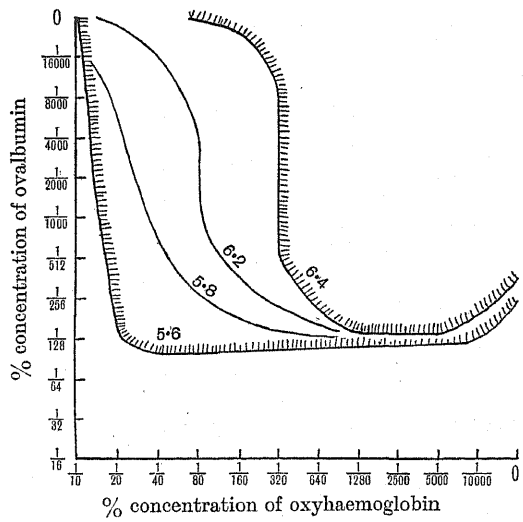


Fig. 4B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

The salt may, however, also affect both the electrostatic and the proper protective effects of the protein. As shown in the previous paper, the minimum protective actions of ovalbumin and gelatin are in the neighbourhood of the isoelectric point, whilst that of oxyhaemoglobin is at about p_H 6, somewhat below the isoelectric point. Mixtures of ovalbumin and gelatin behave in

additive fashion, the protective effect of each accentuating that of the other, with one exception. When very small quantities of gelatin are present, very slightly more ovalbumin is required to protect than when no gelatin is present. This may be due to the effect of a very small quantity of gelatin in the surface layer of the particles, quite inadequate in itself to produce any appreciable protective action, so altering the surface forces that the quantity of ovalbumin adsorbed becomes decreased (see Fig. 2B).

Systems of oxyhaemoglobin and gelatin, or of oxyhaemoglobin and ovalbumin also in general behave additively. The results obtained with these two systems are very similar to each other (see Figs. 3B and 4B). This similarity is particularly marked, as, in the presence of salt, high concentrations of ovalbumin exercise a protective action, even at the isoelectric point, although the minimum at this point is rather more marked than in the case of gelatin. Since, in the absence of salt, ovalbumin precipitates at the isoelectric point even in high concentrations, this result implies a certain amount of peptisation by the sodium chloride, similar to that previously noted in respect to oxyhaemoglobin at p_H 6·5. In the previous paper, the observations on ovalbumin in the absence of salt were not recorded, so that peptisation was not noted. The most satisfactory explanation of the anomalous behaviour of oxyhaemoglobin in the presence of salt, as recorded in the previous paper and confirmed in this one, is that the proper protective effect is minimal not at the true isoelectric point, but at about one unit of p_H below. This shift in the minimum of proper protective effect may or may not be accompanied by a corresponding change in the apparent isoelectric point in the presence of sodium chloride, similar to that noted by Michaelis and Szent-Györgyi [1920] in the case of caseinogen.

The general results in the two systems, oxyhaemoglobin-gelatin and oxyhaemoglobin-ovalbumin, are in harmony with the principles enunciated above, namely, that the proteins independently affect the electric charge, and also independently and additively exercise their proper protective effects. In accordance with the curious effect of salt on the protective action of ovalbumin, it is found that the similar peptising action of salt on oxyhaemoglobin is not inhibited by the presence of ovalbumin, although it seems to be inhibited by the presence of moderate quantities of gelatin.

Precipitation and protection of colloidal gold. Anomalous effects of the type referred to above in connection with oxyhaemoglobin and gum benzoin were more marked when this protein was used with gold. This was referred to in the previous paper. The further complication arises here, that, as mentioned in the experimental section of this paper, the degree of colour change and the extent of precipitation do not necessarily run parallel in the presence of protein. When the precipitation results for two proteins *plus* gold are examined, they are found broadly to conform with the principles enunciated above in the case of gum benzoin, with the modification necessitated by the existence of the anomalies exhibited in the case of oxyhaemoglobin. When the colour change results are examined, they are also found to be in accordance with the above

principles. The lack of parallelism between colour change and precipitation is most marked in the presence of gelatin on the acid side of the isoelectric point, where considerable colour change without flocculation takes place, whilst in

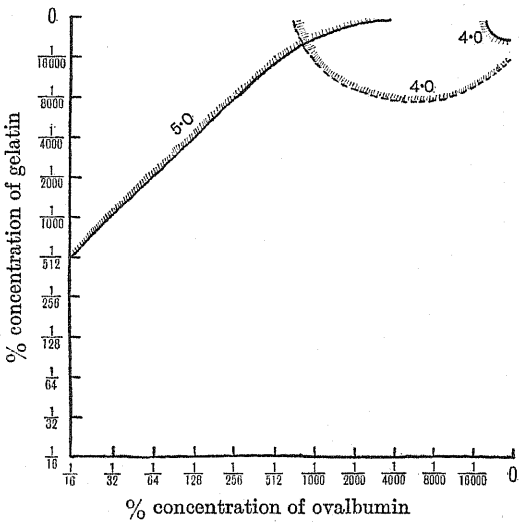


Fig. 5A. Action of mixtures of gelatin and ovalbumin on gold sol at various [H·].

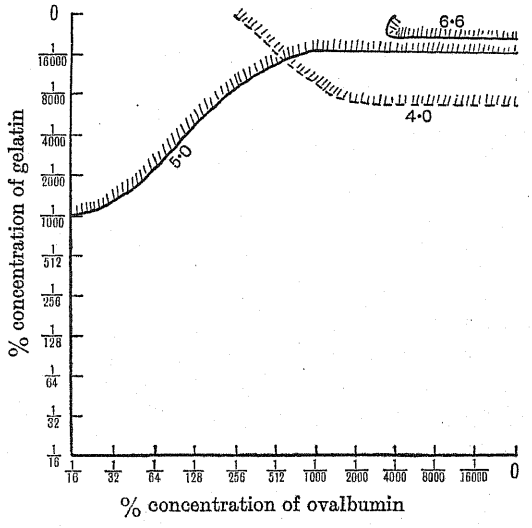


Fig. 5B. Protective action of mixtures of gelatin and ovalbumin on gold sol at various [H·] in presence of 1·25 % NaCl.

the neighbourhood of the isoelectric point of the protein, complete flocculation is observed without equally complete colour change. These remarks are true of mixtures both in the presence and absence of salt.

It was suggested that the probable explanation lay in the small adsorption

of the protein on the gold, and the poor protective action of these proteins on the acid side as compared with that on the alkaline side of the isoelectric point suggests that this small adsorption may be characteristic of the protein cations.

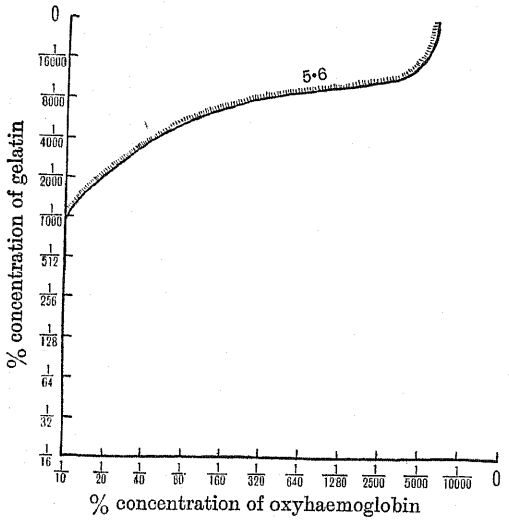


Fig. 6A. Action of mixtures of gelatin and oxyhaemoglobin on gold sol at various [H·

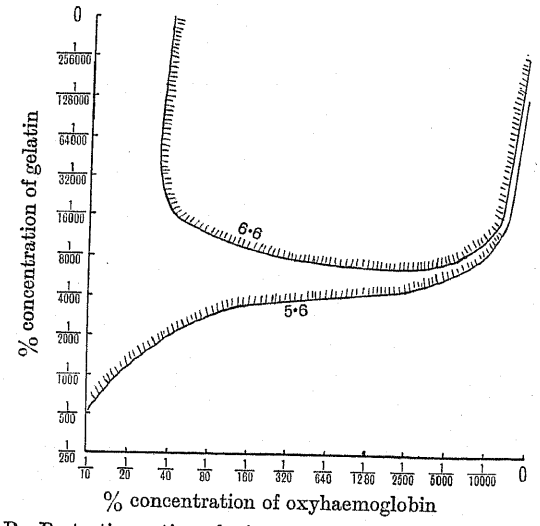


Fig. 6B. Protective action of mixtures of gelatin and oxyhaemoglobin on gold sol at various [H·] in presence of 1·25 % NaCl.

The proper protective effect of the proteins will be measured by the quantity required to prevent precipitation when the gold particles are discharged and the protein molecules are in the neutral condition. The much greater protective action when the p_H is somewhat greater or less than corresponds to the isoelectric point must be regarded as the combined effect of the proper protective

effect of the protein along with stabilisation due to the existence of like charges. On this basis we may consider for example the results obtained with oxyhaemoglobin and gelatin at p_H 5·6 (see Figs. 6 A and 6 B). In the absence of salt,

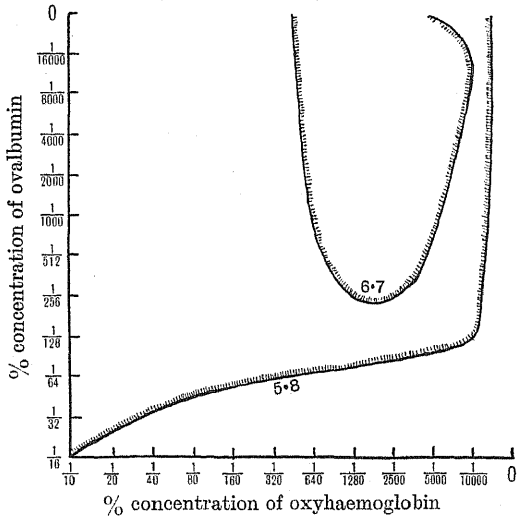


Fig. 7A. Action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various [H·].

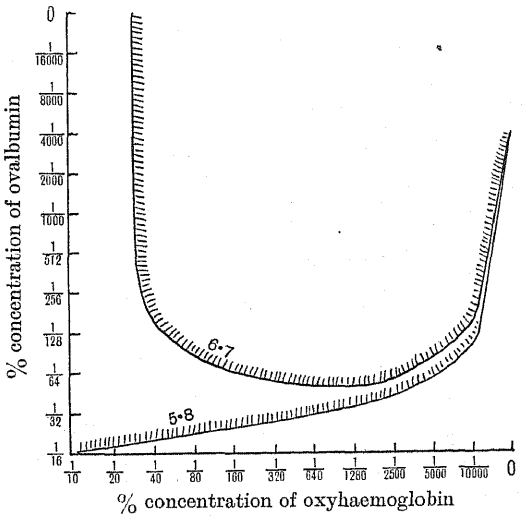


Fig. 7B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various [H·] in presence of 1·25 % NaCl.

there is no precipitation with gelatin alone. In the presence of a sufficient concentration of oxyhaemoglobin, however, precipitation occurs with zero or very small concentrations of gelatin. But with larger concentrations of gelatin the negatively charged gelatin ions reinforce the negative charge on the particles and in this way prevent discharge by the positively charged oxyhaemoglobin.

As the concentration of oxyhaemoglobin increases up to a point, rather more gelatin is required to prevent precipitation. In the presence of salt and absence of oxyhaemoglobin gelatin protects the gold at a concentration of 1/128,000 % and over. At a somewhat lower p_H gelatin does not protect at 1/8000 % concentration. It seems probable that the protective action of gelatin at p_H 5·6 is accentuated by the negative charge borne by the protein at this p_H. When, however, small quantities of oxyhaemoglobin are present, the positive charge on this protein will tend to neutralise the negative charge on the gelatin. The protection of the colloid will then have to be effected largely by the proper

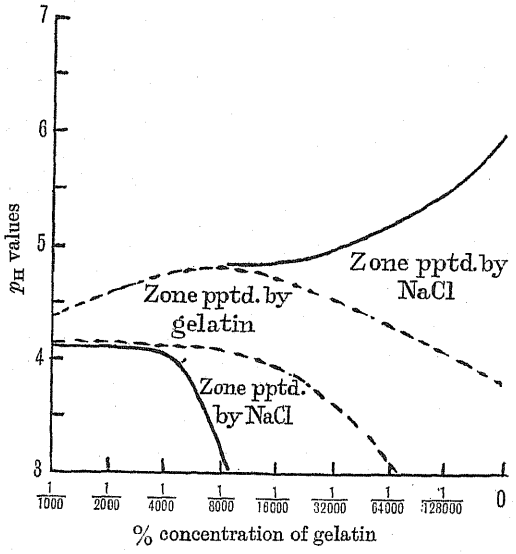


Fig. 8. Action of gelatin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin p_H 4·7.

protective effect of the gelatin assisted by the comparatively small proper protective effect of the oxyhaemoglobin, the latter on the acid side of the isoelectric point. Thus, protection does not take place in the presence of 1/10,000 % oxyhaemoglobin until the concentration of gelatin exceeds 1/8000 %, and this quantity of gelatin increases as the concentration of oxyhaemoglobin increases. The concentration of gelatin never exceeds 1/500 % even when the concentration of oxyhaemoglobin is as high as 1/10 %. The limiting factor here may be the proper protective effect of the gelatin. The results obtained with the other mixtures of proteins conform to the same principles. In the case of gelatin and ovalbumin the results obtained are similar to those obtained with this mixture of proteins and gum benzoin (Figs. 5 A and 5 B) whilst those obtained with ovalbumin and oxyhaemoglobin are similar to those obtained with gelatin and oxyhaemoglobin with gold (Figs. 7 A and 7 B).

SUMMARY.

1. Experiments have been performed on the precipitation of colloidal gold and gum benzoin by mixtures of ovalbumin and gelatin, ovalbumin and oxyhaemoglobin, and of oxyhaemoglobin and gelatin at various hydrogen ion concentrations.

2. Observations have also been made on the protective action of these mixtures at various hydrogen ion concentrations against precipitation of the lyophobic colloids by 1·25 % sodium chloride.

3. It appears that the results previously obtained with the single proteins can be extended to apply to the above mixtures. If the effect of a protein on the charge of the lyophobic particles is distinguished from its proper protective effect, the two proteins would appear to exercise each of these two influences additively.

REFERENCES.

Hopkins, Kermack and Spragg (1931). *Biochem. J.* **25**, 829.
Michaelis and Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.
Prideaux and Howitt (1931). *Biochem. J.* **25**, 391.

CCI. INFLUENCE OF PROTEINS AND PROTEIN DERIVATIVES ON THE ENZYMIC HYDROLYSIS OF STARCH BY MALT DIASTASE.

By BRONISŁAW FILIPOWICZ.

From the Biochemical Laboratory, Faculty of Medicine, the University, Warsaw.

(*Received September 28th, 1931.*)

Much of the work published by earlier authors on the activation or inactivation of amylase by various substances, such as amino-acids, amines, amides, proteins, *etc.*, is vitiated by the fact that possible variations in the p_H of the medium due to the introduction of the substances examined were not taken into consideration. The same applies to other factors influencing the activity of this enzyme, such as purity, temperature, *etc.*

Euler and Josephson [1926] found that the addition of glucose or laevulose retarded the velocity of enzymic amylolysis whilst addition of amines or amino-acids to such systems accelerated the reaction. Sherman, Caldwell, and Adams [1926] considered that the activating effect of amino-acids was due to their inhibiting action on the autolysis of the enzyme. Przyłęcki and Majmin [1931] have shown that proteins can combine with, and consequently possibly activate the substrate, the combination between starch and proteins being dependent on the p_H of the medium. It follows that amylolysis in presence of protein should depend quantitatively on the p_H of the medium, and in the present paper work on this aspect of the problem will be described.

Experimental.

Methods.

Solutions of Merck's amylase (Diastase absolut) were prepared separately for each series of determinations by dissolving 0·1 g. of amylase in 100 cc. of 0·1 % NaCl, and filtering after 30 min. Solutions of Merck's soluble starch or dextrin were prepared by gradual addition of starch or dextrin paste to boiling water, boiling a further 15 min., and, after cooling, diluting to the requisite volume. Two buffer solutions were used: Walpole's acetate mixtures below p_H 4·5, and Sørensen's phosphates above p_H 4·5. p_H was determined colorimetrically in some cases but chiefly potentiometrically. Reducing sugars obtained from starch were determined by Bertrand's method, using 60 cc. of solution and 20 cc. each of Bertrand's reagents. Merck's ovalbumin (6 g.) was dissolved in 100 cc. of water, and filtered after 30 min.; the filtrate had

p_H 7·27. The solutions were freed from protein before determination of sugar by addition of 30 cc. of a mixture of 200 cc. of 4·5 % $ZnSO_4$ and 40 cc. of 0·1 *N* NaOH, heating for 3 min. at 100°, and filtering. 60 cc. of the filtrate were taken for determination of sugar. Peptone solutions (Poulenc) were prepared in an analogous manner; their p_H was 4·83. Gelatin (Coignet Père et Fils) was dissolved in water at 90°; the solutions had p_H 5·89.

A. *Enzymic hydrolysis of starch at varying* p_H.

To a number of 200 cc. flasks 15 cc. of 3 % starch, 15 cc. of the appropriate buffer solutions, 1 cc. of 0·1 % amylase and 29 cc. of water were added. The flasks were then left for 1 hour at 30°, and maltose was determined. The results (Table I) indicate that the optimal p_H is, under the given conditions, 4–4·5. In more acid solutions the activity of amylase rapidly falls, being practically zero at p_H 3·0.

Table I. *Enzymic hydrolysis of starch at varying* p_H*.

p_H	mg. maltose	p_H	mg. maltose
3·0	5·1	6·0	59·5
3·5	57·4	6·5	54·1
4·0	65·8	7·0	48·2
4·5	64·0	7·5	37·5
5·0	62·2	8·0	25·3
5·5	59·8	—	—

* Results in all Tables are the mean of 4 observations.

B. *Action of amylase in the presence of ethyl alcohol.*

1. *Velocity of reaction in various concentrations of alcohol.* A series of flasks was prepared containing 30 cc. of 1·5 % starch, 10 cc. of buffer solutions p_H 5·6, 18 cc. of aqueous alcohol, so as to obtain concentrations of 1, 5, 10, 15 and 20 % of alcohol in the whole system, and 2 cc. of 0·1 % amylase. The systems containing 15 and 20 % of alcohol were turbid, owing to partial precipitation of starch. All flasks were kept 2 hours at 30°. The results (Table II) show that percentage retardation of reaction is directly proportional to the concentration of alcohol.

Table II. *Influence of conc. of alcohol on amylolysis at* p_H *5·6.*

% alcohol	mg. maltose	% retardation
0	89·6	—
1	87·5	2·3
5	83·5	6·8
10	78·3	12·6
15	71·2	20·5
20	64·6	27·9

2. *Velocity of reaction in 5 % alcohol at varying* p_H. A number of flasks were prepared, containing 15 cc. of 3 % starch, 15 cc. of buffer solutions, 3·125 cc. of alcohol, 25·875 cc. of water, and 1 cc. of 0·1 % amylase. The systems were left 1 hour at 30°. The results (Table III) are similar to those

obtained in the absence of alcohol, maximum reaction taking place at p_H 4·5. Reaction is retarded at p_H above 4·5 by alcohol to an extent increasing with p_H.

Table III. *Amylolysis in 5 % alcohol at varying* p_H.

p_H	mg. maltose: With alcohol	mg. maltose: Without alcohol	% retardation due to alcohol
3·5	51·5	52·8	2·6
4·5	58·4	60·6	3·7
6·0	48·5	55·3	12·5
7·5	23·2	32·3	28·7

3. *Reaction in alcoholic media with varying concentrations of starch.* Two series of flasks were prepared, one containing 0·5 and the other 1·5 % starch; in both series alcohol was present in concentrations of 0, 1, 5, 10, 15 and 20 %. The systems, consisting of 30 cc. of starch, 10 cc. of buffer solution (p_H 6·5), 19 cc. of aqueous alcohol or water, and 1 cc. of 0·1 % amylase, were kept at 30° for 2 hours. The results (Table IV) indicate that percentage retardation of reaction due to alcohol is practically independent of the concentration of starch taken. At the same time the yield of maltose is about 30 % greater using 1·5 than 0·5 % starch, both in the presence and absence of alcohol.

Table IV. *Amylolysis at various concentrations of alcohol and starch.*

% alcohol	0·5 % starch: mg. maltose	0·5 % starch: % retardation	1·5 % starch: mg. maltose	1·5 % starch: % retardation
0	41·0	—	54·7	—
1	39·5	3·6	53·8	1·6
5	36·2	11·7	51·1	6·5
10	34·7	15·3	47·1	13·9
15	31·9	22·2	41·7	23·7
20	28·7	30·0	35·4	35·2

4. *Comparison with dextrin.* The retarding action of alcohol may be due to partial precipitation of starch. Should this be the case, the retarding action of alcohol should be absent from systems containing dextrin, which is not precipitated by the concentration of alcohol used. The following experiment shows that this is not the case. A number of flasks each containing 30 cc. of 1·5 % starch or dextrin, 10 cc. of buffer solution (p_H 6·5), 12·5 cc. of 96 % alcohol or water, 5·5 cc. of water and 2 cc. of 0·1 % amylase were left 2 hours at 30°, after which maltose was determined. Since the dextrin used contained some reducing sugars, the value found for systems such as the above to which 2 cc. of water had been added in place of amylase, was deducted from the reducing sugar content of the above systems. The results obtained were substantially the same for starch as for dextrin; thus 86·5 mg. of maltose were produced from starch in the absence of alcohol and 62·9 mg. in its presence, a retardation of 26·8 %. The corresponding values for dextrin are 37·0 and 27·6 mg., and the retardation is 24·3 %.

5. *Action of alcohol on amylase.* 0·1 g. of amylase and 0·1 g. of NaCl were dissolved in 100 cc. of 20 % alcohol, and kept 1 hour at 30°, together with a

similar aqueous solution. A number of systems, containing 30 cc. of 1·5 % starch, 10 cc. of buffer solution (p_H 6·5), 12·5 cc. of alcohol or water, 2·5 cc. of water, and 5 cc. of aqueous or 20 % alcoholic amylase solution, were kept 1 hour at 30°, and reducing sugar was determined. The activity of amylase previously subjected to the action of alcohol was smaller than was that of amylase dissolved in water, in both alcoholic and aqueous solutions (81·5 and 84·7 mg. of maltose produced respectively, as compared with 109 and 111·5 mg. in the presence of aqueous amylase).

Four solutions of amylase were now prepared, as follows:

Amylase I. 0·4 g. of amylase and 0·4 g. of NaCl in 100 cc. of 20 % alcohol;

Amylase II. The same, in 5 % alcohol;

Amylase III. The same, in 1 % alcohol;

Amylase IV. The same, in water.

These 4 solutions were kept for 22 hours at 30°. Solutions I and II remained clear, whilst solutions III and IV contained a flocculent precipitate. Four series of flasks were prepared, each containing 30 cc. of 1·5 % starch, 10 cc. of buffer solution (p_H 6·5), 19·5 cc. of water, and 0·5 cc. of amylase solution I, II, III, or IV. The maltose produced after 1 hour at 30° was 89·5 mg. by amylase I, 85 mg. by amylase II, 88·3 by amylase III, and 74·6 mg. by amylase IV. Alcohol therefore inhibits the inactivation of amylase in solution.

Three volumes of water were now added to amylase I, II and IV, yielding amylase I′ (0·1 %, in 5 % alcohol), II′ (0·1 %, in 1·25 % alcohol), and IV′ (0·1 % in water). These solutions were kept 2 hours at 30°, and 2 cc. of each were added to similar systems as in the preceding experiment. Maltose determined after 2 hours at 30°, amounted to 74·6 mg. with amylase I′, 66·6 mg. with amylase II′, and 59·2 mg. with amylase IV′.

The same experiment was repeated with amylase solutions I′, II′ and IV′, kept a further 24 hours at 30° before addition to the systems, the maltose after 3 hours at 30° now being respectively 102, 68·1, and 50·1 mg.

It appears that the protective influence of alcohol on amylase becomes accentuated with duration of contact, the relative acceleration of action of amylase in 5 % alcohol amounting after 22 hours to 15 %, after 24 hours to 20 %, and after 48 hours to 100 %.

C. *Action of amylase in the presence of proteins and of their derivatives.*

1. *Influence of ovalbumin at varying* p_H. The action of amylase was examined in freshly prepared systems containing ovalbumin buffered to p_H 3·5, 4·5, 6 and 7·5. A number of 200 cc. flasks were prepared, containing 15 cc. each of 3 % starch, buffer solution, and 6 % ovalbumin solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase. All systems were kept 1 hour at 30°, after which 20 cc. of Bertrand's solution I were added to the control flasks in order to interrupt the reaction, whilst 30 cc. of Hagedorn-Jensen solution

were added to the flasks containing albumin, which was then coagulated by the method described above. The systems were filtered, and 60 cc. of the filtrates were taken for determination. Maltose was determined by Bertrand's method, and the result was multiplied by 1·5 in order to obtain the value for the whole system. The results (Table V) indicate that the action of ovalbumin is a function of p_H; thus at p_H 3·5 ovalbumin retards reaction by about 5 %, whilst at higher p_H it accelerates reaction progressively to 43·4 % at p_H 7·5.

Table V. *Action of ovalbumin at varying* p_H.

	mg. maltose produced		
p_H	With protein	Without protein	% acceleration
3·5	50·3	52·8	−4·9
4·5	63·8	60·6	+5·3
6·0	61·7	55·1	+11·9
7·5	46·1	32·1	+43·4

2. *Influence of ovalbumin in 5 % alcohol.* Similar systems to those described in the preceding experiment were set up, with the difference that 3·125 cc. of alcohol were added in place of a similar volume of water, so that these systems contained 5 % of alcohol. The p_H, temperature and duration of reaction were as before. The results (Table VI) were of the same order as in the absence of alcohol; the absolute yield of maltose was, however, smaller in all cases (retardation due to alcohol), whilst the retarding or accelerating influence of ovalbumin was relatively greater (12·3 % retardation at p_H 3·5, and 77 % acceleration at p_H 7·5).

Table VI. *Action of ovalbumin at varying* p_H *in 5 % alcohol.*

	mg. maltose produced		
p_H	With protein	Without protein	% acceleration
3·5	45·1	51·5	−12·3
4·5	63·1	58·4	+ 8·1
6·0	56·4	48·5	+16·3
7·5	41·8	23·2	+77·0

3. *Influence of gelatin at varying* p_H. A number of flasks were prepared, containing 15 cc. each of 3 % starch, phosphate buffers, and 4 % gelatin solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase. The systems were buffered to p_H 5, 6, 7 and 8, and were kept 1 hour at 30°, after which maltose was determined as usual. The results obtained (Table VII) are not of the same

Table VII. *Action of gelatin at varying* p_H.

	mg. maltose produced		
p_H	With gelatin	Without gelatin	% acceleration
5	59·0	54·9	7·3
	80·2	77·9	3·0
6	65·6	59·3	10·8
7	49·3	38·4	29·0
	47·0	41·9	12·7
8	40·0	28·8	60·1

order of accuracy as in other experiments, owing to the unreliable values given by Bertrand's method in the presence of gelatin, and to the circumstance that experiments at each p_H were conducted separately. It is, however, obvious that gelatin accelerates reaction increasingly from p_H 5 to 8.

4. *Influence of gelatin in 20 % alcohol.* The systems consisted of 15 cc. each of 3 % starch, phosphate or acetate buffers, and 4 % gelatin or water, 12·5 cc. of alcohol, 1·5 cc. of water and 1 cc. of 0·1 % amylase. The systems were at p_H 3·5, 5, 5·8, 6·5 and 7, and were maintained at 30° for 1 hour. As in the preceding experiment, each value is strictly valid only for the corresponding control. Gelatin acts similarly in alcoholic and aqueous systems, its effect being however more marked in the former. In particular, very considerable retardation (64·6 %) is observed at p_H 3·5 (Table VIII), whilst at higher p_H acceleration increases from about 10 % at p_H 5 to about 70 % at p_H 7.

Table VIII. *Action of gelatin at varying* p_H *in 20 % alcohol.*

	mg. maltose produced		
p_H	With gelatin	Without gelatin	% acceleration
3·5	13·6	38·7	−64·6
5·0	48·0	41·8	+13·3
5·8	47·7	37·6	+26·8
6·5	46·4	29·5	+57·3
7·0	30·0	17·2	+72·6

5. *Influence of peptone.* A number of systems consisting of 15 cc. each of 3 % starch, phosphate buffers to p_H 4·5, and 4 % Witte's peptone or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30°, and maltose was determined as usual. A mean value of 66·0 mg. was obtained in the presence of peptone, and of 60·3 mg. in its absence, representing an acceleration of 9·3 % due to peptone.

6. *Influence of glycine.* In the above experiments the effect on the action of amylase of addition of colloidal proteins or protein derivatives has been studied; the effects obtained have been ascribed by certain authors to protective colloidal action. In order to ascertain to what extent this view is justified, experiments were performed on the action of crystalloid protein derivatives.

(*a*) *Glycine.* A number of systems containing 15 cc. each of 3 % starch, buffer solutions, and 4 or 8 % glycine solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30° after which maltose was determined. The concentration of glycine in the systems was 0·133 M and 0·266 M. The results (Table IX) indicate that glycine has a similar action to that of the colloidal protein derivatives studied previously, and that the effect obtained increases with the concentration of glycine.

That the action of glycine is not exerted directly on the substrate was shown by experiments in which similar systems to the above, but without amylase, were taken; no production of maltose took place.

Table IX. *Action of glycine at varying* p_H.

Systems as in Table V, except that 4 or 8 % glycine is taken in place of 6 % ovalbumin.

	1 % glycine			2 % glycine		
	mg. maltose produced			mg. maltose produced		
p_H	With glycine	Without glycine	% acceleration	With glycine	Without glycine	% acceleration
3·5	51·1	54·2	− 6·4	—	—	—
4·5	69·4	61·0	+13·8	65·2	50·8	+28·4
6·0	65·5	54·3	+21·0	63·0	45·4	+38·8
7·5	48·3	35·1	+38·0	48·4	30·4	+59·2

(*b*) *Influence of temperature on action of glycine.* Systems of the same composition as in the above experiments, and at p_H 3·5, 4·5, 6 and 7·5, were studied at 18, 30, 38 and 50°. The time of reaction at 18° was 4 hours, and the yields of maltose were divided by 4 in order to obtain comparative results with systems kept 1 hour at 30, 38 and 50°. The results obtained (Table X) indicate that the absolute velocity of reaction increases up to 50°; at 60° the activity of amylase is smaller than at 50°. This is in accordance with Chrząszcz's finding [1923] that at the optimal p_H amylase exhibits maximal activity at 55°. At lower p_H values the velocity of reaction appears to be independent of temperature. At higher p_H the accelerating effect due to glycine increases with temperature.

7. *Influence of sodium aspartate.* Similar experiments to those with glycine were performed, substituting 15 cc. of 9·4 % sodium aspartate for the same volume of 4 % glycine, so that the molar concentration (0·133) was the same as for glycine. At p_H 4·5, 97·6 mg. of maltose were produced in the presence of sodium aspartate, and 71·0 mg. in its absence, an acceleration of 37·4 %,

Table X. *Influence of temperature on enzymic hydrolysis of starch in the presence of glycine.*

Systems as in Table V, except that 4 % glycine is taken in place of 6 % ovalbumin. Time of reaction: 1 hour.

		mg. maltose produced		
Temperature	p_H	With glycine	Without glycine	% acceleration
18°	3·5	19·0	20·3	− 6·4
	4·5	28·0	23·6	+14·4
	6·0	24·2	20·0	+21·0
	7·5	17·6	12·6	+39·7
30°	3·5	54·9	58·5	− 6·2
	4·5	74·6	65·7	+13·7
	6·0	69·4	56·7	+22·5
	7·5	53·6	38·6	+38·8
38°	3·5	60·9	65·8	− 7·4
	4·5	89·5	78·5	+14·0
	6·0	84·1	66·9	+25·7
	7·5	64·6	35·5	+82·0
50°	4·5	125·0	107·0	+16·8
	7·5	81·0	31·0	+161·0

whilst at p_H 6·0 the corresponding figures are 63·8 and 51·0 mg., and 21·8 % acceleration.

D. *The active group of amino-acids.*

1. *The carboxylic group.* Sherman and co-workers [1927], who investigated the influence of acids on the action of pancreatic amylase, found that at the optimal p_H the presence of acetic, phosphoric, boric and citric acids did not affect the velocity of hydrolysis. The effects of addition of oxalic and acetic acids will now be described. Systems containing 15 cc. each of 3 % starch, phosphate buffer p_H 7·5, and 9·36 % potassium oxalate solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30°, and reducing sugar was determined. Oxalate retards reaction under these conditions by about 8 %, the yield of maltose being 45·2 mg. in the absence of oxalic acid, and 41·6 mg. in its presence. The molar concentration of potassium oxalate was 0·133.

The concentration of sodium acetate taken in similar systems was 0·266 *M*, so that the concentration of carboxylic groups was the same in both cases. Acetate produces a relatively greater retardation of reaction, *viz.* 29 %, the yield of maltose falling from 45·2 to 32·1 mg.

2. *The amino-group.* The influence of amino-groups has been investigated by Caujolle and Molinier [1930], who found that at 50° aliphatic amines inactivated pancreatic, salivary and malt amylases. The hydrochlorides of these amines, on the other hand, activated pancreatic and salivary amylases, but were without effect on malt amylase. These authors do not, however, state whether the p_H of their systems was affected by the addition of amines, so that it is possible that the effects observed were due to change in p_H. Sherman and Naylor [1922] also report an inactivation of amylase by aliphatic and aromatic amines.

(*a*) *Guanidine carbonate.* This was taken at concentrations of 0·013 and 0·133 *M*, in systems consisting of 15 cc. of 3 % starch, 15 cc. of hydrochloric acid solution, 7·5 cc. of 0·54 or 5·4 % guanidine carbonate solution or water, 21·5 cc. of water and 1 cc. of 0·1 % amylase solution. The p_H of the systems was adjusted to 4·5 and 6 by addition of hydrochloric acid of the requisite strength, and maltose was determined after 1 hour at 30°. The yield of maltose in the control systems was 76 mg., whilst in the presence of guanidine only traces of sugar were found.

(*b*) *Diethylamine.* Diethylamine in concentrations of 0·133 *M* similarly totally inhibits reaction at p_H 7·5.

(*c*) *Asparagine.* Asparagine (1 %) in similar systems totally inhibits reaction at p_H 4·5.

(*d*) *Methylamine hydrochloride.* This salt (0·133 *M*) totally inhibits reaction at p_H 7·5, whilst at p_H 4·5 the yield of maltose is 58·0 mg. in its presence, as compared with 71·1 mg. in its absence, a retardation of 18·4 %. Sørensen's phosphate buffers were used in these experiments.

(*e*) *Urea.* 0·133 *M* urea has only a small retarding influence, the yield of maltose at p_H 4·5 being 71·0 mg. in its presence, and 75 mg. in its absence, a retardation of 5·4 %.

(*f*) *Choline hydrochloride.* This salt, at concentrations of 0·133 *M*, exerts a slight activating influence, the yield of maltose being 63·6 mg. in its presence, and 61·0 mg. in its absence, an activation of 4·3 %.

Discussion.

The above results indicate that proteins and their derivatives are capable of exerting a profound influence on the velocity of enzymic hydrolysis of starch, to an extent dependent on the p_H of the medium. At p_H lower than the optimal (p_H 4·5) the reaction is in all cases retarded, whilst at and above p_H 4·5 it is accelerated to an extent proportional to the alkalinity of the system.

These results appear to be connected with Przyłęcki and Majmin's [1931] work on the combination of proteins and their derivatives with starch. These authors found that the stability of such complexes varied inversely with the p_H of the medium, combination being irreversible in acid solutions. The starch in such complexes is possibly more resistant to the action of amylase than is free or loosely combined starch, and this might explain the inhibitory action of the substances examined in systems at p_H 3·5.

Activation of reaction at higher p_H may be due to activation of the enzyme or of the substrate, or to protection of the enzyme from some anticatalyst formed during the reaction; which of these factors is actually responsible for the effect observed it is not possible, on the basis of the experimental material available, to state. Since, however, the action of protein substances on the enzyme itself has not been examined, these explanations must be regarded as being purely speculative.

The magnitude of the effect observed appears to be proportional to the active concentration of amino-acid units present, as is shown by Table XI.

Table XI.

Substance	% concentration	p_H	% acceleration
Ovalbumin...	1·5	4·5	5·3
Gelatin	1·0	5·0	6·6
Peptone	1·0	4·5	9·0
Glycine 0·133 *M*	1·0	4·5	13·8
Glycine 0·266 *M*	2·0	4·5	28·4
Sodium aspartate 0·133 *M*	2·3	4·5	37·4

Percentage acceleration is twice as great at p_H 4·5 in presence of 2 % as in that of 1 % glycine. At other p_H the results are qualitatively of the same order, but the proportionality between concentration and activation is not so exact.

The action of amino-acid groups is not due to either the amino- or the carboxyl groups taken separately; the addition of carboxylic acids is either without effect or exerts a retarding action, whilst amines inhibit reaction to an

extent proportional to the basic character of the amino-group. These findings are in accordance with those of earlier workers in this field.

The action of proteins and of their derivatives appears to be due to their amino-acid units, and not, as has been supposed by certain authors, to their protective colloidal character. The results obtained possess a considerable biological significance, in that they indicate that the action of amylase within the living organism will be modified by the protein substances which are present in abundance, and this applies in particular to its action at varying p_H. Thus, in the intestinal digestion of carbohydrates it would follow that the velocity of hydrolysis should be greater when proteins are simultaneously undergoing digestion than when these are absent.

In the presence of ethyl alcohol, retardation of reaction is observed both in the presence and in the absence of protein substances, and this retardation is, at a given concentration of starch, roughly proportional to the concentration of alcohol, and is greater in alkaline than in acid media. On the whole, the action of alcohol is greater in more dilute than in more concentrated solutions of starch.

Were the inhibitory action of alcohol to be due to denaturation of the enzyme, it might be expected that this effect would be obtained in quite low concentrations of alcohol, as the concentration of enzyme is small. Experiment showed, however, that this is not the case, as the activity of the enzyme was little affected by 1 hour's contact with 20 % alcohol, and after 48 hours of contact alcoholic enzyme solutions were more active than were similar aqueous solutions.

The inhibitory action of alcohol may be due to some extent to diminution in the degree of dispersion of starch, solutions of which exhibit a marked turbidity on the addition of alcohol. This explanation would agree well with the observed proportionality between the concentration of alcohol and the percentage inhibition of reaction, as well as with the observation that the yield of maltose, at the same concentration of enzyme, is proportional to the concentration of the substrate only with small concentrations of starch. On the other hand, experiments with dextrin show that a practically identical inhibition of reaction is obtained as with starch, in spite of the fact that dextrin is aggregated by alcohol to a far less extent.

The possibility remains that alcohol acts on the enzyme-substrate complex, by eluting particles of starch from the surface of the enzyme, which is consequently isolated from contact with the substrate to a greater or less extent.

The effect of the addition of protein to systems containing alcohol is of the same order as in non-alcoholic systems, but is numerically greater, both at p_H at which protein retards and at which it accelerates reaction. The absolute values are, however, in all cases lower than in the absence of alcohol.

SUMMARY.

1. Ethyl alcohol retards the reaction of hydrolysis of starch by malt diastase to an extent proportional at p_H 5·6 to the concentration of alcohol. The optimal p_H in the presence of alcohol is the same as in its absence, *viz.* 4·5. Percentage retardation of reaction due to alcohol is greater the more the p_H of the system exceeds 4·5.

2. Similar results are obtained taking dextrin in place of starch.

3. The activity of 20 % alcoholic amylase solutions is greater than is that of similar solutions not containing alcohol, after keeping up to 48 hours.

4. Ovalbumin, gelatin, peptone, glycine and aspartic acid retard at p_H below the optimal (p_H 4·5), and accelerate reaction at p_H higher than 4·5, to an extent increasing with the alkalinity of the medium. In the case of glycine, the relative acceleration is greater the higher the temperature.

5. The action of protein substances is not due either to the carboxylic or to the amino-group separately, as both carboxylic acids and amines retard the reaction.

This research was undertaken at the suggestion and under the direction of Prof. St. J. Przyłęcki, to whom the author wishes to express his indebtedness for his help and supervision.

REFERENCES.

Caujolle and Molinier (1930). *Compt. Rend. Soc. Biol.* **104**, 294.
Chrząszcz (1923). *Biochem. Z.* **142**, 417.
Euler and Josephson (1926). *Z. physiol. Chem.* **152**, 31.
Przyłęcki and Majmin (1931). *Biochem. Z.* **240**, 98.
Sherman and Naylor (1922). *J. Amer. Chem. Soc.* **44**, 2956.
—— Caldwell and Adams (1926). *J. Amer. Chem. Soc.* **48**, 2954.
—— —— and Dale (1927). *J. Amer. Chem. Soc.* **49**, 2597.

CCII. SPERMINE IN HUMAN TISSUES.

By GEOFFREY ARTHUR HARRISON.

From the Department of Chemical Pathology, St Bartholomew's Hospital.

(*Received October 1st, 1931.*)

Although the structure of spermine has been elucidated by the work of Dudley, Rosenheim and their colleagues [1924, 1925, 1926], the physiological significance of this interesting base remains completely obscure. Originally observed in, and suspected as being specific to, human semen, subsequent investigation showed it to be a widely distributed constituent of mammalian tissues, both male and female.

That it is not essential to the process of fertilisation was made extremely probable by the failure of Dudley and Rosenheim [1925, 2] to detect it in the semen of the bull. An explanation of this striking difference from human semen is advanced in the present communication, which is concerned mainly with the distribution of the base in human tissues; it was considered desirable to make this survey in order to compare the results with those of Dudley and Rosenheim [1925, 2] for other mammalian tissues, and in the hope that the information obtained might eventually contribute to an understanding of the biological significance of the base.

Method of isolation.

The base was separated by the steam-distillation method [Dudley, Rosenheim and Rosenheim, 1924]. A number of minor modifications were introduced so that a description may be of value.

The organs, which were obtained from the *post mortem* room, were the seat of various pathological lesions. By working up one gland at a time it was hoped originally that variations due to different diseases might be revealed, at any rate in the case of the larger glands. In nearly all cases (for exceptions, see below), however, the yield of spermine was so low that it was necessary to use 5 kg. of minced organ to work quantitatively; for this reason the attempt to isolate spermine from a single organ had to be abandoned.

With the exceptions noted below, the following procedure was finally adopted. The organs were cleaned by dissection, minced, and stored under absolute alcohol, until 5 kg. or more of a particular organ had been collected. The fluid was then strained off, the alcohol was removed by distillation and the residue was added to the mince. The weight of the mince *plus* residue was determined, and the final yield was expressed as mg. of spermine phosphate per 100 g. of this material. These were obviously not the yields per 100 g. of fresh

organ (they were a little higher in most cases), but were satisfactory for comparative purposes. In the case of liver, however, all the observations were made on organs as observed at autopsy; in the case of lung and brain also it was sometimes possible to obtain enough minced organs to avoid storage under alcohol.

The 5 kg. of minced organ were added to 10 litres of boiling tap-water, and thoroughly mixed. After the addition of 30 cc. of glacial acetic acid and further stirring, the mixture was again boiled and kept simmering for half an hour. The coagulated protein was allowed to settle, and the supernatant fluid was syphoned off and filtered on a large Büchner funnel. The bulky mass of coagulated protein was transferred to a press, and as much fluid as possible was squeezed out and then passed through the Büchner funnel. The combined filtrates were then evaporated to about 500 cc. The contents of the pan were transferred, without filtration, to a glass vessel, together with a little wash-water. After introducing 25 cc. of glacial acetic acid, a saturated aqueous solution of normal lead acetate was added till there was no further immediate precipitate. The mixture was either filtered on a Büchner funnel or (more conveniently) passed through a Sharples centrifuge. The excess of lead was removed from the filtrate by cautious precipitation with 50 % *v/v* sulphuric acid. The lead sulphate was removed by filtration or centrifuging. The filtrate was made slightly alkaline to litmus by adding flake sodium hydroxide, and concentrated to about 400 cc. Ammonia was thus largely removed, but no spermine was lost.

The concentrate was transferred, with sufficient wash-water to make a total volume of 500 cc., to a 3 litre round-bottom flask of resistance glass, and 50 g. of flake sodium hydroxide were added. A grease-mark was made on the flask to indicate the level of the fluid which was maintained at constant volume. The mixture was steam-distilled in the usual way, a Kjeldahl trap being inserted in the exit tube to prevent loss of the solution by frothing or spitting. Frothing was often troublesome, and it was generally essential to regulate the distillation carefully for a few minutes, and sometimes to add a little vaselin.

Distillation was continued until there was no longer any smell of ammonia, and until the distillate was only slightly alkaline to litmus (usually 2 to 6 litres of distillate). In some cases the fluid which came over gave a positive Dragendorff's reaction, but it was shown that this was due to other bases and never to spermine, provided that the contents of the distilling flask were not allowed to become concentrated. (To make certain of this point, this "10 % distillate" was on many occasions worked up separately, but no spermine was recovered.) When a positive Dragendorff's reaction was obtained, distillation was continued until it became negative. This preliminary distillation from 10 % sodium hydroxide was a valuable means of getting rid of most of the ammonia (formed by the action of the alkali), and of some of the "lower bases."

The contents of the distilling flask were allowed to cool a little, and then a further 200 g. of flake sodium hydroxide were added. The steam-distillation was again started, and continued vigorously until samples of the distillate no

longer gave a positive Dragendorff's reaction. Care had to be taken that the contents of the flask did not become diluted by condensed steam. To ensure that the whole of the base had been distilled over, a further 50 g. of sodium hydroxide were added and the distillation was continued. This was repeated if necessary until Dragendorff's reaction was negative. As a rule some 5 to 15 litres of distillate were collected. This was made acid with concentrated hydrochloric acid and taken to complete dryness by distillation *in vacuo* or by evaporation on the water-bath. (Completion of the drying and the volatilisation of the excess of hydrochloric acid were facilitated by adding absolute alcohol in the last stages of evaporation.)

The very crude spermine hydrochloride thus obtained was dissolved in the minimum of distilled water and filtered with suction through a large filter-paper to remove a small amount of greasy pigmented matter, which was washed with distilled water. The combined filtrate *plus* washings was acid, and on making slightly alkaline to litmus by the cautious addition of 40 % sodium hydroxide, a further small pigmented precipitate was generally obtained and was filtered off after adding decolorising charcoal.

The final solution of spermine hydrochloride was treated with a concentrated solution of sodium picrate (picric acid 23 g., *N*/1 NaOH 100 cc., water 500 cc.) until no further precipitate could be obtained. After standing at room temperature for a few hours (generally overnight) the crude spermine picrate was filtered off, dried and weighed. (The mother-liquor, after concentration, as a rule yielded no significant quantities of spermine picrate.)

The dry crude picrate was suspended in sufficient acid alcohol (concentrated HCl 7 cc., water 43 cc., alcohol to 200 cc.) to dissolve it after heating on the boiling water-bath for about 10 minutes. For each 1 g. of picrate 15 to 25 cc. of the acid alcohol were generally required. The hot solution was poured into about 10 volumes of acetone, the glass vessel being washed out with a further few cc. of hot acid alcohol. The mixture was kept until the precipitate clumped and was then filtered with suction. The precipitate was washed with acetone until free from picric acid.

This precipitate of crude spermine hydrochloride rapidly deliquesced; the funnel was therefore promptly connected up with another filter-flask, a small volume of water was added and the solution sucked through. To this solution the calculated amount (weight of picrate × 0·236) of diammonium hydrogen phosphate, dissolved in a small volume of warm water, was added. An equal volume of absolute alcohol was added to the mixture which was kept at room temperature overnight and then filtered. The precipitate was dissolved in the minimum of boiling water and the solution treated with charcoal and filtered hot.

To the final filtrate which was colourless, a few drops of phenol red were added, and (if necessary) 10 % ammonia until the reaction was p_H 7·0 to 7·2 (warm brown tint). Crystallisation of spermine phosphate often started rapidly but was completed by adding $\frac{1}{3}$ vol. of absolute alcohol. After standing overnight at room temperature, the precipitate of spermine phosphate

was filtered off, dried and weighed. The mother-liquors after addition of alcohol to 50 % and to 25 % respectively, in the last two stages, were always worked up, but seldom yielded significant amounts of spermine phosphate.

Notes on method.

When the first filtrate, after removal of protein, contains appreciable quantities of fatty material in suspension, it is necessary to introduce one or more extractions with ether.

Treatment with lead may be omitted, as in the original description [Dudley *et al.* 1924], but if it is, considerable quantities of ammonia are formed owing to the action of sodium hydroxide on the protein, and frothing is likely to be much more troublesome. Filtration of the lead precipitates is in some cases very tedious or difficult; centrifuging is a great improvement.

If the steam-distillate is treated with phosphoric acid as described by Dudley *et al.* [1924], and is then evaporated to a small volume, it becomes very acid owing to loss of ammonia. If the reaction is not readjusted, spermine phosphate will generally not crystallise out at all. If concentration is effected before adjustment of reaction with phosphoric acid, spermine phosphate crystallises out, but in the writer's experience the yield is lower than in the method above described, possibly owing to the effect of the other phosphates on the solubility of spermine phosphate. The preliminary distillation after addition of sodium hydroxide to 10 % largely eliminates this difficulty.

The conversion of the crude hydrochloride (obtained by acidification and evaporation to dryness of the steam-distillate), into picrate, into hydrochloride and then into phosphate, may at first sight appear a tedious and unnecessarily complicated procedure, but gives a comparatively clean and pure preparation with more certainty, in the writer's opinion, than the original method of Dudley *et al.* [1924]. Incidentally it may be noted that the weight of picrate has generally been some four to ten times the weight corresponding to the yield of phosphate finally obtained (except in the case of the prostate). This presumably is due in part at any rate to the presence of picrates of bases other than spermine [*cf.* Dudley, Rosenheim and Starling, 1927].

A better yield was obtained by first precipitating crude spermine phosphate in the presence of 50 % alcohol and then recrystallising from 25 % alcohol, than by precipitating it initially with $\frac{1}{3}$ vol. of alcohol.

Finally, decoloration with charcoal of extracts, which are generally deep brown, allows phenol red to be used and therefore a close adjustment of reaction to be made.

Conversion of spermine into phosphate via *picrate and hydrochloride.*

In order to gain some idea of the magnitude of losses which might be expected in the process of conversion of crude spermine hydrochloride into picrate, into hydrochloride and finally into phosphate, the following experiment was performed on pure spermine phosphate[1].

0·4315 g. of spermine phosphate was suspended in 10 cc. of water and sufficient sodium hydroxide solution was added to cause solution (12 drops of 40 % NaOH). The solution was then treated with sodium picrate, and the subsequent stages were carried through exactly as described for a tissue extract.

The 0·4315 g. of phosphate gave 0·92 g. of picrate (theory, 0·953 g.), 0·285 g. of hydrochloride, 0·394 g. of "crude" phosphate, and 0·370 g. of once recrystallised phosphate. That is to say approximately 86 % of the original phosphate was finally recovered. Since this experiment further experience

[1] Kindly presented by Dr Dudley.

has been obtained in handling spermine salts, and it is now considered that this recovery should be regarded as a minimum with ordinary care.

Yields.

The yield of spermine, as phosphate, in mg. per 100 g. of tissue is shown in Table I. The result of each experiment is given, except in the case of liver on which eleven sets of analyses were made. The results of Dudley and Rosenheim [1925, 2] for certain animals are quoted for comparison.

Wrede [1926] separated spermine as the chloroaurate from several organs of man, cow and bull. It is probable that his chloroaurate contained small quantities of bases other than spermine; on the other hand the maximum weight of organ employed by him was 2 kg., which would tend to give low yields. His results (recalculated as spermine phosphate) for human testis (0·75), muscle (2·25), brain (3·75), spleen (5·75), liver (12·5), pancreas (13·5) and semen (95 mg. per 100 g.) are of the same order as the writer's (see Table I).

Table I.

	Spermine as phosphate, mg. per 100 g.	
Tissue	In man	In animals (Dudley and Rosenheim)
Semen	90, 100, 117, 260*	0 (bull)
Testis	1·2, 9·3	6 (bull)
Prostate	130	—
Ovary	—	14 (cow)
Pancreas	16·1	25 to 30 (ox)
Liver	2·3 to 11·4	16 (ox)
Kidney	5·0, 5·9	15 (ox)
Spleen	3·0, 8·0	11 (ox)
Lung	3·3, 3·3, 4·7	7 (ox)
Heart	1·5, 2·3	—
Muscle	—	12 (calf), 4 (sheep) 2 (ox), 2 (horse)
Brain	1·8, 2·0, 3·1	7 (ox)
Thymus	—	5 (calf)
Adrenal	—	5 (ox)
Thyroid	—	3 (ox)
Bone marrow	0	—
Blood	0, 0	0 (ox), 0 (sheep)†
Serum	0	0 (ox)
Sputum	0, 0	—
Faeces (dried)	Trace, trace	—
Milk	—	0 (cow)
Egg	—	0 (hen)

* Result of Rosenheim. † Personal finding, 7791 cc. sheep's blood.

Notes on Table. With the following exceptions 5 kg. or slightly more of tissue were used for each analysis: testis, 975 and 1551 g.; prostate, 1594 g.; pancreas, 3361 g.; whole blood, 400 and 600 cc.; serum, 250 cc.; sputum, 380 and 200 cc.; bone marrow, 15 cc.; dried faeces, 450 and 190 g. The testes and prostates took over a year to collect. Further details as regards the yields from semen are as follows: (*a*) 18·3 cc. collected under alcohol; steam-distillation method applied to precipitate; yield 90 mg. per 100 cc. (*b*) 4·9 cc. of fresh seminal plasma (spermatozoa removed by centrifugation); direct separation of Böttcher's crystals; yield 100 mg. per 100 cc. (*c*) 60 cc. semen, pooled, stored in ice-room for several weeks without preservative; steam-distillation from alkali applied direct to untreated semen; yield 117 mg. per 100 cc. In addition spermine phosphate (Böttcher's) crystals have several times been separated from whole semen, both fresh and stale, by careful adjustment of reaction, but not quantitatively.

Discussion of results.

The yields obtained for the majority of tissues need no special comment. Spermine is present in almost every tissue of man just as in animals [Dudley and Rosenheim, 1925, 2], but in most organs of man it is in slightly lower concentration.

The two points of chief interest arising out of the present investigation are first, the large yield of spermine from the prostate, and second the presence of the base in the faeces.

The prostate consists approximately of two parts of muscle and one part of glandular tissue. If it may be assumed that most of the spermine is derived from the glandular portion of the gland, then the yield of 130 mg. per 100 g. of whole gland represents a yield of 300 to 400 mg. per 100 g. of glandular tissue; in other words it makes it likely that the main part, if not the whole, of the spermine of human semen is derived from the prostatic secretion.

Bull's semen contains no spermine [Dudley and Rosenheim, 1925, 2]; the bull does not possess a true functional prostate, but only a rudimentary collection of compound acinar glands which are regarded as the equivalent of the human prostate. This may account for the absence of spermine from the semen of the bull. Wrede [1926] recovered 70 mg. of spermine chloroaurate from 600 g. of bull's "prostate," which is equivalent to a yield of 3·5 mg. of spermine phosphate per 100 g. of tissue. Whether the "Rinder prostata" used by Wrede was rudimentary prostate or seminal vesicles or both, is immaterial; his yield was so low (of the same order as found in other organs of the body) that it is compatible with the absence of spermine from bull's semen.

The writer's observation (see notes on Table I) that fresh human seminal plasma yields at least as much spermine as whole semen, makes it unlikely that the spermatozoa are a significant source of the base.

Fürbringer [1886] stated that human prostatic secretion contained an organic base, the phosphate of which constituted "Böttcher's crystals" or "prostatic crystals." It is only recently [Rosenheim, 1924] that "Böttcher's crystals" from human semen were shown conclusively to consist of spermine phosphate, but it is curious that no worker seems to have explored further Fürbringer's statement that in man "Schreiner's base" (as spermine was then called) was derived from the prostate. Fürbringer [quoted by Von Frisch, 1899] found that the prostatic fluid squeezed out of prostates obtained *post mortem* contained Böttcher's crystals in 56 cases (90 % of cases examined). Fürbringer failed to recover Böttcher's crystals directly from fresh prostatic secretion obtained by massage of the gland during life, but he found that the crystals could be made to appear by adding ammonium phosphate. He concluded therefore that the prostatic secretion supplied the base, and that the phosphoric acid was derived from other components of the semen. He explained his finding of Böttcher's crystals in *post mortem* prostatic fluid by assuming that autolysis of epithelium supplied the phosphoric acid.

Although only some 1 to 5 drops of prostatic fluid are usually obtained by massage during life, it is easy to confirm Fürbringer's experiment of producing Böttcher's crystals by adding ammonium phosphate, though the crystals are of the characteristic shape only if the final reaction of the mixture is close to p_H 7·0. The writer has several times confirmed Fürbringer's work both by adding ammonium phosphate and by adding phenol red (as indicator) and dilute phosphoric acid to p_H approximately 7·0 to expressed prostatic fluid. In both cases the addition of a little alcohol (about 0·1 vol.) facilitated crystallisation. In one instance the addition of acetic acid (and phenol red) produced a few crystals of the typical lenticular shape, so that Fürbringer's failure to obtain Böttcher's crystals from prostatic fluid expressed during life may in part at any rate have been due to failure to secure the correct reaction.

It has been claimed that the prostatic secretion has an activating action on the spermatozoa [see Fürbringer, 1886, and Von Frisch, 1899]. There is no doubt of the effect of adding prostatic secretion to spermatozoa which have previously not come into contact with such secretion; the spermatozoa remain active for a much longer period in the presence of the prostatic fluid. Conversely, human spermatozoa, which have been separated from semen by centrifuging and washing with 0·85 % NaCl, do not remain motile for nearly so long as the spermatozoa left in suspension in the (diluted) seminal plasma (an observation several times confirmed by the writer). But neither of these observations of necessity proves that the normal function of the prostatic secretion is to activate the spermatozoa, though they support the idea.

It was however considered to be worth while to try the action of different concentrations of spermine phosphate in physiological saline on the motility of human spermatozoa separated from semen and washed three times with 0·9 % NaCl. Equal volumes of the suspension of spermatozoa and of spermine phosphate in physiological saline were mixed; control observations were made without the spermine phosphate. It was found that spermine phosphate in a final concentration of 19, 13, 1·3 and 0·13 mg. per 100 cc. had no activating action on the spermatozoa as compared with the controls.

The discovery of spermine in human faeces may be of significance. Just as putrescine in faeces is derived from the putrefaction of the arginine (ornithine) component of proteins, so spermine might be derived from the putrefaction of an appropriate component of proteins, although no parent substance, capable of yielding it, has yet been observed in protein hydrolysates. In a preliminary observation the writer has obtained traces of spermine after incubating a mixture of a platinum loopful of normal faeces with 2000 cc. of 2 % Witte's peptone for 63 hours, and 7 days respectively, under both aerobic and anaerobic conditions. Control tests, in which inoculation with organisms was omitted, were negative. This subject is under investigation.

SUMMARY.

1. A description of the steam-distillation method of separating spermine from tissues is given, modifications having been introduced to obtain the base as phosphate more readily in a relatively pure state.

2. A study of the distribution of the base in human tissues shows that it is virtually the same as that found in animals by Dudley and Rosenheim.

3. The spermine content of the human prostate is remarkably high, and probably most of the spermine of human semen is derived from this organ.

4. The absence of spermine from the semen of the bull is probably to be explained by the absence of a true functional prostate in that animal.

5. Spermine occurs in human faeces.

The author is indebted to the Medical Research Council for a grant towards the expenses of this work.

REFERENCES.

Dudley and Rosenheim (1925, 1). *Biochem. J.* **19**, 1032.
—— —— (1925, 2). *Biochem. J.* **19**, 1034.
—— —— and Rosenheim (1924). *Biochem. J.* **18**, 1263.
—— —— and Starling (1926). *Biochem. J.* **20**, 1082.
—— —— —— (1927). *Biochem. J.* **21**, 97.
Fürbringer (1886). *Berl. klin. Woch.* **23**, 476.
Rosenheim (1924). *Biochem. J.* **18**, 1253.
Von Frisch (1899). Nothnagel's Pathologie u. Therapie, **19**. (II. Theil, III. Heft.) 11.
Wrede (1926). *Z. physiol. Chem.* **153**, 291.

CCIII. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

III. EFFECT OF ALKALI ON THE SENSITIVITY TOWARDS INSULIN OF ALBINO RATS.

By ALEXANDER HYND and DOROTHY LILLIE ROTTER.

From the Physiology Department, University of St Andrews.

(*Received October 5th, 1931.*)

In a previous paper [Hynd and Rotter, 1931] it has been shown that the effect of a carbohydrate-free diet on the sensitivity to insulin varies with the species of animal employed for the experiment. Further, as the results obtained pointed to the conclusion that animals became resistant to insulin on diets which were capable of causing a lowering of the alkali reserve, the experiments now described were carried out with the object of obtaining further support for this view.

As Hawkins [1924] has shown that rabbits and guinea-pigs show a great variation in acid-base equilibrium, while rats, on the other hand, exhibit considerable constancy both in p_H and CO_2 content of the whole blood, the rat was the animal selected for our experiments. Moreover, as we have found that piebald rats, though fed on the usual stock diet, vary considerably in their sensitivity to insulin, all the experiments described in this paper were carried out on standard albino rats of 120–160 g. body weight. The basis of the diets employed was bread and milk for the carbohydrate-fed animals, and cheese for the carbohydrate-free animals. To these diets were added varying amounts of different salts as detailed below, and after certain periods the animals were tested for their response to insulin. The experiments were so arranged that the effect of the salts on the response to insulin could be observed simultaneously in carbohydrate- and non-carbohydrate-fed animals, as well as in control animals on a normal diet without the addition of any salts.

Experimental.

(1) *Effect of sodium bicarbonate on cheese-fed rats.* Seven normal rats, which had been receiving the ordinary stock diet of bread and milk, were transferred to a diet of cheese to which was added 0·12 g. $NaHCO_3$ per g. of cheese. After 1 week the animals were tested with insulin: none was resistant.

The diet was continued for another week with 4 of the rats, and again all responded to the injection.

(2) *Effect of potassium bicarbonate on cheese-fed rats.* An experiment similar to the above was carried out with 8 rats, the sodium bicarbonate being replaced by an equivalent amount (0·14 g.) of potassium bicarbonate. On testing the animals with insulin after 1 week on this diet, all were resistant, but after 2 weeks they were found to have lost their resistance.

As the animals in this and in the preceding experiment lost weight, the second dose of insulin, being proportional to the body weight at the time of injection, was somewhat smaller than the first dose, so that the loss of resistance to insulin is all the more striking.

(3) *Effect of sodium bicarbonate on rats previously rendered resistant to insulin by a cheese diet.* Seventeen normal rats were fed on cheese for a fortnight and proved to be resistant to the usual dose of insulin. Each animal was then fed with cheese containing 0·12 g. $NaHCO_3$ per g. After 2 days on this diet, one animal was tested with insulin and found to be partially resistant. After 3 days, 4 rats were tested and found non-resistant; after 4 days, 7 other animals were tested and found non-resistant, and after 5 days, 5 other rats were also found non-resistant to insulin.

After 3 days, therefore, all the rats responded to insulin, although the behaviour was not quite typical. In almost every case the animals became cold and limp, and convulsions if they occurred were not of a violent type. Moreover, administration of glucose did not bring about a rapid recovery, and 3 of the animals died.

Similar results were obtained in an experiment with mice.

(4) *Effect of potassium bicarbonate on rats previously rendered resistant to insulin by a cheese diet.* Eight rats rendered resistant to insulin by a cheese diet were then given cheese and an amount of potassium bicarbonate equivalent to the sodium bicarbonate used in the preceding experiment.

After 5 days on this diet all the animals were still resistant. Four of the rats were continued on this diet for 9 days and still failed to respond.

Similar results were obtained in an experiment with mice.

(5) *Comparison of effect of (a) $NaHCO_3$ and (b) $KHCO_3$ on the blood-sugar, muscle- and liver-glycogen, and liver-fat of cheese-fed rats.* Owing to the striking difference obtained in the response to insulin after 5 days with potassium bicarbonate, as described in Exp. (4), compared with that after 3–5 days with sodium bicarbonate in Exp. (3), it seemed advisable to know the blood-sugar level, the muscle- and liver-glycogen content, and the liver-fat content of the animals in the two series.

Accordingly, 4 rats were kept on a cheese diet for 15 days and assumed to be resistant to insulin, as it seemed inadvisable actually to test the animals, lest the glycogen distribution might be affected thereby. As in 32 experiments we have encountered only 3 rats which under this treatment did not become resistant to insulin, the assumption seemed justifiable. Two of the rats were then fed on cheese containing 0·12 g. $NaHCO_3$ per g. for 4 days, and the other two on cheese containing the equivalent amount of $KHCO_3$ per g. for the same

period. That is, the $NaHCO_3$ pair when analysed would have lost their resistance to insulin, while the $KHCO_3$ pair would still be insensitive.

The analyses (Table I) show no significant differences.

Table I.

Rat	Diet	Response to insulin	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %
1	Cheese + $NaHCO_3$	+	0·175	1·0	1·4	5·4
2	,, ,,	+	0·145	0·93	1·8	4·2
	Average...	+	**0·16**	**0·965**	**1·6**	**4·8**
3	Cheese + $KHCO_3$	−	0·122	0·79	0·87	3·7
4	,, ,,	−	0·16	0·94	2·60	4·9
	Average...	−	**0·141**	**0·865**	**1·73**	**4·3**

(6) *Effect of sodium bicarbonate on carbohydrate-fed rats.* (*a*) *Small dose of $NaHCO_3$* per os. Five rats from the stock cages were kept without food for 9 hours. Each animal was then given bread and milk containing 0·5 g. $NaHCO_3$, and 15 hours later was tested for its response to insulin.

Four of the rats showed typical symptoms, though the effect, compared with that on a control animal receiving no alkali, was somewhat delayed in its development. Further, after the administration of glucose the animals did not recover well, and one of them, although repeatedly injected with glucose, never recovered sufficiently to be able to take food and died in about 6 hours after the insulin injection. One rat did not react, and, as this animal was still resistant to insulin after the alkali had been continued for a week, it was considered to be abnormal.

(*b*) *Large dose of $NaHCO_3$* per os. Three rats were each given bread and milk containing 2 g. $NaHCO_3$, and as in the preceding experiment then tested with insulin. All responded in a normal fashion, though the effect was slightly delayed.

(*c*) *Subcutaneous injection of $NaHCO_3$.* Two rats on a bread and milk diet were injected subcutaneously with 2 cc. of 8 % solution of sodium bicarbonate, and ½ hour later given insulin. In both, typical symptoms ensued very rapidly, as in 1 hour after the insulin injection glucose had to be administered to relieve the convulsions, whereas a control animal which received a corresponding dose of insulin showed no outward sign of hypoglycaemia until 1½ hours later.

(*d*) *Effect of alkali when continued for 1–2 weeks.* To the ordinary daily ration of bread and milk 0·5 g. $NaHCO_3$ was added and fed to each of 4 rats. Four other rats each received an equivalent amount of $KHCO_3$, and 3 rats were given a larger amount of $NaHCO_3$, *viz.* 2 g. each daily.

The animals took a sufficient quantity of food and remained almost steady in weight. Meyer and Cohn [1911] state that the addition of potassium bicarbonate to the diet of a rat causes the animal to lose weight rapidly, while with sodium bicarbonate rats put on weight. No difference in this respect was detected in the animals fed on the two alkalis. The examination of the urine

collected was rendered of rather doubtful value, as, though the animals took the diet well, there was always a considerable amount of food scattered. The urine in every case was found to be strongly alkaline and to reduce Benedict's solution. In the case of the rats receiving the larger amount of $NaHCO_3$, small amounts of acetone bodies were detected.

After 1 week on these diets, the animals were injected with insulin and all responded in a more or less normal manner. The rats which had received $KHCO_3$ differed from those treated with the sodium salt in that, though they had more violent convulsions, they made more rapid and more complete recoveries. On the other hand, the animals which had received sodium bicarbonate made slow recoveries from the effects of the insulin injection, relapses were frequent, and 2 of the rats were found dead the following morning.

The experiment with the rats on the potassium bicarbonate was continued for a second week and the animals were re-tested with insulin. A normal response again resulted in every case, though the development of symptoms was slightly delayed.

Animals treated similarly to those just described were also employed for determinations of blood-sugar, muscle- and liver-glycogen and liver-fat. The average values obtained are given below in Table II, and for comparison values are quoted from our previous paper [Hynd and Rotter, 1930]. In addition, there is included the result of the following experiment. A rat, weighing 160 g. at the beginning of the experiment, was fed for 1 week on bread and milk to which were added 2 g. $NaHCO_3$ daily. A control animal of equal weight was fed for 1 week on bread and milk, so adjusted that the two rats were eating practically the same amount of food. Both were then starved for 24 hours before making the analyses.

Table II.

Daily diet contains	Duration of experimental feeding (weeks)	Period of fasting (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %
0·5 g. $NaHCO_3$	2	0	0·15	0·77	3·4	—
Control	2	0	0·16	0·77	3·4	3·4
2 g. $NaHCO_3$	1	0	0·14	0·78	2·6	2·4
Control	1	12	0·13	0·70	2·5	3·7
2 g. $NaHCO_3$	1	24	0·11	0·90	1·1	4·2
Limited ration	1	24	0·135	0·81	1·1	4·5

(7) *Effect of ammonium chloride on carbohydrate-fed rats.* Each of 4 rats was fed on bread and milk containing 1–2 g. NH_4Cl per day's ration. As the animals objected to this diet, the experiment was continued for only 4 days. At the end of this period the rats were tested for their response to insulin. Three of the animals were resistant but showed the early transitory sprawling similar to that previously described for rats rendered insensitive to insulin by a cheese diet. The fourth rat reacted to insulin. This animal had very severe convulsions, from which it made only a partial recovery though injected with the

usual amounts of glucose. It died suddenly 5½ hours after the administration of the insulin.

(8) *Effect of alkali on rats previously rendered resistant to insulin by feeding ammonium chloride.* Four rats were rendered resistant to insulin by feeding ammonium chloride as described in the preceding experiment. Two of the animals were then given bread and milk containing sodium bicarbonate (1·2 g. $NaHCO_3$ each daily). The other two received the equivalent of potassium bicarbonate. After 2 days the animals were no longer resistant to insulin. All responded in a normal manner, the symptoms being similar in both sodium- and potassium-fed animals.

Discussion.

Several years ago Murlin and Kramer [1913] suggested that mobilisation of glycogen is favoured by an acid medium and its storage by an alkaline medium. The results of the foregoing experiments are in full accord with this hypothesis and prove beyond question that the acid-base balance is an important factor in determining the effectiveness of the pancreatic hormone.

The resistance to the action of insulin, which rats develop when maintained on a carbohydrate-free diet, can be altered by the addition of alkalis to the diet. Animals previously rendered resistant to insulin by a diet of cheese display a more or less normal sensitivity after 3 days' treatment with sodium bicarbonate. On the other hand, an equivalent amount daily of potassium bicarbonate has little, if any, effect even though continued for 9 days. Nevertheless, analysis shows (Table I) that the blood-sugar, the muscle- and liver-glycogen and also the liver-fat of a rat giving a response to insulin after the sodium bicarbonate treatment are practically identical with those of a rat still resistant to insulin after the ingestion of an equivalent amount of alkali in the form of potassium bicarbonate. Again, while sodium bicarbonate prevents a cheese diet from bringing about resistance to insulin, potassium bicarbonate fed along with cheese produces resistance in a shorter period (1 week) than does cheese alone (12–14 days). Continued feeding of potassium bicarbonate, however, eventually destroys the resistance to insulin, so that it appears that this difference between the two salts is only temporary.

Bunge [1873] concluded that the ingestion of large amounts of potassium led to an increased output of sodium, so that though large amounts of salts were being fed, the output might exceed the intake and a lowering of alkali reserve result. However, even though the high intake of potassium were continued, a point would soon be reached at which the body would retain its sodium, so that the alkali reserve would rise. Our results are explicable on this theory. Feeding potassium bicarbonate would produce a temporary period of low alkali reserve, during which the animals would be resistant to insulin, and this would be followed by a gradual increase in the alkali reserve with a consequent return of a more or less normal response. Feeding sodium bicarbonate on the other hand, would lead to a steady increase in the alkali reserve, as no

drastic elimination of potassium results, and consequently no resistant period would be observed. Richards, Godden and Husband [1927] find, however, that large doses of potassium do not lead in growing pigs to an increased elimination of sodium, or to an increased retention of potassium, but cause an increased excretion of calcium. Excess of sodium, on the other hand, was followed by retention of calcium, so that the Na/K ratio of a diet may have a very considerable influence on the calcium assimilation. The fact that Brougher [1927] has shown that insulin increases the blood-calcium may, therefore, be of importance. However, though certain experiments of Geiger and Kropf [1930] led them to form the opinion that a diet with a high calcium content favoured carbohydrate metabolism—a view supported by clinical experience—decreased calcium assimilation may play only a minor part in bringing about the resistance to insulin observed in our experiments. A simpler, although not complete, explanation is that owing to the diuretic effect of the potassium salt there is a smaller retention of total base when potassium bicarbonate is given than when sodium bicarbonate is the alkali used.

Further, it is to be noted that only on the acid-forming cheese diet does this difference between the effects of sodium bicarbonate and potassium bicarbonate become apparent. When rats are fed on the normal diet of bread and milk the only difference detected in the effect of the two salts on the response to insulin is that, as already pointed out, the animals receiving sodium bicarbonate are less liable to have convulsions and recover more slowly and less completely from the insulin symptoms. Probably in this series of experiments sodium bicarbonate increases the alkali reserve more quickly than does the potassium salt, but this point was not investigated as we were concerned mainly with the effect on the response to insulin.

As indicated in the experimental part, with one exception all the rats on the carbohydrate-diet with the addition of either sodium bicarbonate or potassium bicarbonate reacted to the insulin injection. Our results, therefore, are contrary to the findings of Goldblatt [1927], who reported that alkali inhibits the action of insulin. In his experiments, this worker used rats and also a rabbit which was admittedly abnormal. As he did not state the amount of alkali fed to each animal, it remains uncertain whether we have repeated his conditions, and in any case Goldblatt did not report on the action of insulin on rats fed for more than one day with alkali. Neither after a small dose nor a large dose of sodium bicarbonate *per os*, for one or more days, did we find the action of insulin inhibited, and administration of alkali subcutaneously definitely hastened and accentuated the response to insulin. We also failed to confirm the remarkable and almost complete disappearance of glycogen from both liver and muscle noted by Goldblatt to occur as the result of feeding sodium bicarbonate to rats for 3–8 days. Our values for the liver- and muscle-glycogen in rats fed with sodium bicarbonate are somewhat lower than those of controls, but the diminution is not striking, and we attribute the slight decrease to the fact that animals do not take the normal amount of bread and

milk when sodium bicarbonate is added to it. In other words, we are merely studying, in such experiments, the effect of slight undernutrition. This is indicated clearly by the results summarised in Table II, from which we conclude that alkali feeding does not reduce the liver- or muscle-glycogen content of carbohydrate-fed rats.

Goldblatt's contention that alkalis interfere with the action of insulin appears to receive support from the work of Haldane, Wigglesworth and Woodrow [1924, 2]. It is to be noted, however, that, though these workers found the ingestion of alkali to cause hyperglycaemia and glycosuria after less than half the normally tolerated dose of glucose, they did not actually demonstrate resistance to insulin. Moreover, the prolonged hyperglycaemia observed is not necessarily an index of defective insulin action [Cajori, Crouter and Pemberton, 1925], and further, Haldane and his co-workers draw attention to the fact that the symptoms of faulty sugar metabolism do not come on until long after the concentration of sodium bicarbonate in the blood is at its maximum. Goldblatt, further to support his view, also quotes the experiments of Hetényi [1926]. The results obtained by this investigator, however, not only in animal experiments but also with diabetic patients [Hetényi, 1927] point decidedly in the opposite direction, namely that alkali favours the action of insulin. Again Goldblatt's reference to the work of Koehler [1926] is misleading. While it is true that Koehler found huge doses of insulin to have no marked effect in several diabetic patients with marked acidosis (blood-p_H 6·8–7·2), Goldblatt omitted to add that "after alkali in the form of 3 % sodium bicarbonate solution was administered intravenously in just sufficient quantities to return the blood reaction to normal, the blood-sugar decreased very rapidly." Hédon [1925] also, as the result of experiments with a depancreatised dog, came to the conclusion that insulin and sodium bicarbonate are together required to restore the alkali reserve and health in cases of diabetes. Moderate dosage of sodium bicarbonate is also approved of by Campbell and Macleod [1925].

On the other hand, as it has been proved that insulin increases the alkali reserve, it is held by many and emphasised particularly by Joslin, that it is not only unnecessary but harmful to give alkali in diabetes. However, this assumes that the insulin is exerting its effect. If the conditions are such that the action of insulin is weakened in a marked degree, insulin treatment alone evidently will not correct the alkali reserve. In other words, it is essential that the alkali reserve to begin with be not too far reduced, otherwise the secreted or injected insulin will be rendered ineffective.

Further support for the view that the action of insulin is favoured by alkali and inhibited by acid is furnished by our experiments with ammonium chloride. The addition of this salt to the bread and milk diet very quickly produces in rats a severe acidosis, and even though abundant carbohydrate is supplied in the diet, there is established simultaneously a marked resistance to insulin, which, moreover, is easily corrected by replacing the ammonium chloride with either sodium or potassium bicarbonate, although here again the sodium salt

is more effective. At the same time, it must not be forgotten that an ammonium chloride acidosis is accompanied by an excessive excretion of water and also of sodium and potassium ions [Haldane *et al.*, 1924, 1]. Moreover, though the absolute loss of potassium may be less than that of sodium, the relative loss of potassium is much greater than that of sodium. Consequently, the Na/K ratio may be altered greatly. This may have an important effect on the action of insulin, especially as it is here shown that there is, for a time at least, a marked difference in the effects produced by sodium and potassium bicarbonates, both as regards their ability to dispel resistance to insulin and also in the type of insulin response which may be elicited.

In dealing with the resistance to insulin reference should perhaps be made to the well-known fact that fat depancreatised animals are much more difficult to control with insulin than are lean animals. This appears to us not to be due to a hyperglycaemia being maintained by a continuous conversion of fat into carbohydrate [Macleod, 1930], but rather to the tendency which fat animals show to develop acidosis and thereby render the insulin less effective. A similar explanation, that is, a greater tendency to acidity, seems applicable to the so-called resistant types of human diabetes. Lawrence [1927] holds that such cases of diabetes are either infected ones, or complicated by over-activity of the thyroid or suprarenals. While admitting that these glands are recognised antagonists to the pancreas, the conditions indicated are just those which would be accompanied by excessive acidity.

In conclusion it may be pointed out that the results of the present investigation suggest an alternative explanation to that usually given as to why glucose produced endogenously appears less stimulating to insulin production than ingested carbohydrate [Lawrence, 1926]. There is a large body of evidence to support the view that a raised blood-sugar is the normal stimulus to the production of insulin by the pancreas. Why then does the hyperglycaemia occurring in the later stages of starvation, when the metabolism of protein increases [Morgulis and Edwards, 1924], or that which results when a normal animal is maintained on an excess fat diet not stimulate the output of insulin and thereby correct the hyperglycaemic condition? The answer seems evident, for the formation of glucose from sources other than carbohydrate involves the simultaneous production of acid substances. Thus, though the glucose produced furnishes the necessary stimulus to the pancreas and an adequate secretion of insulin occurs, the acid character of the medium will interfere with the effectiveness of the insulin, and, at the same time, as shown by Abderhalden and Wertheimer [1924], will facilitate the action of adrenaline. The net result therefore would be an increased blood-sugar by a further depletion of the glycogen stores. The present tendency to increase the amount of carbohydrate and diminish the amount of fat in the diet of diabetics [Rabinowitch, 1930: Poulton, 1931] is thus shown to be founded on a sound basis. Further, as patients so treated keep well and require much less insulin, the clinical results support the views advanced in this communication.

SUMMARY.

Rats previously rendered resistant to insulin by a cheese-diet soon become responsive after the addition of sodium bicarbonate to the diet, but remain resistant after the ingestion of the equivalent amount of potassium bicarbonate.

Sodium bicarbonate is also more efficient than potassium bicarbonate in preventing the development of resistance to insulin in rats on a carbohydrate-free diet.

This striking difference between the effects of the bicarbonates of sodium and potassium is shown to bear no relationship to the carbohydrate stores of the animal. The acceptance of Bunge's theory of sodium impoverishment by potassium feeding furnishes a possible explanation.

Administration of sodium bicarbonate does not reduce the liver- or-muscle-glycogen of carbohydrate-fed rats, and it favours rather than inhibits the action of insulin.

An ammonium chloride acidosis is accompanied by marked resistance to insulin, which readily disappears after the addition of either sodium bicarbonate or potassium bicarbonate to the diet.

The results, taken as a whole, suggest that, even with abundant carbohydrate to stimulate the production of insulin and presumably with a normal secretion of insulin, the activity of the pancreatic hormone will be interfered with if the medium be too acid.

The clinical importance of the results obtained is discussed.

The authors are indebted to Prof. Herring for his continued interest in this work, and to the Carnegie Trust for research grants.

REFERENCES.

Abderhalden and Wertheimer (1924). *Pflüger's Arch.* **206**, 451.
Brougher (1927). *Amer. J. Physiol.* **80**, 411.
Bunge (1873). *Z. Biol.* **9**, 104.
Cajori, Crouter and Pemberton (1925). *J. Biol. Chem.* **66**, 89.
Campbell and Macleod (1925). Insulin, p. 110. (Williams and Wilkins, Baltimore.)
Geiger and Kropf (1930). *Arch. exp. Path. Pharm.* **147**, 272.
Goldblatt (1927). *Biochem. J.* **21**, 991.
Haldane, Wigglesworth and Woodrow (1924, 1). *Proc. Roy. Soc. Lond.* B **96**, 1.
—— —— —— (1924, 2). *Proc. Roy. Soc. Lond.* B **96**, 15.
Hawkins (1924). *J. Biol. Chem.* **61**, 147.
Hédon (1925). *Compt. Rend. Acad. Sci.* **180**, 545.
Hetényi (1926). *Klin. Woch.* **5**. 800.
—— (1927). *Z. ges. exp. Med.* **57**, 409.
Hynd and Rotter (1930). *Biochem. J.* **24**, 1390.
—— —— (1931). *Biochem. J.* **25**, 457.
Koehler (1926). *J. Biol. Chem.* **67**, xlv.
Lawrence (1926). *Quart. J. Med.* **20**, 69.
—— (1927). *Brit. J. Exp. Path.* **8**, 1.
Macleod (1930). *Brit. Med. J.* i, 918.
Meyer and Cohn (1911). *Z. Kinderheilk.* **2**, 360.
Morgulis and Edwards (1924). *Amer. J. Physiol.* **68**, 477.
Murlin and Kramer (1913). *J. Biol. Chem.* **15**, 365.
Poulton (1931). *Proc. Roy. Soc. Med.* **24**, 1291.
Rabinowitch (1930). *Can. Med. Assoc. J.* **23**, 489.
Richards, Godden and Husband (1927). *Biochem. J.* **21**, 971.

CCIV. NOTES ON OIL DEVELOPMENT IN THE SEED OF A GROWING PLANT.

By JOHN VARGAS EYRE.

(*Received October 9th, 1931.*)

Some years ago, in connection with problems arising in the Linen Industry, an examination was made of certain biological changes which take place in the flax plant during the processes of growth and of ripening. These had to do with the study of so-called "quality" of fibre for spinning and with "quality" of seed for special purposes. In the course of these investigations, results were obtained of considerably wider interest than those in view at the time, and although reference has been made to these investigations [Armstrong, 1924] the results have not hitherto been published. Through the courtesy of the Linen Industry Research Association it is now possible to give an account of this work.

The only previous work bearing on this question was that described by Ivanoff [1912] who examined the biological aspect of oil accumulation. This work was repeated under far more exactly controlled conditions by Eyre and Fisher [1915] who confirmed the general conclusions of Ivanoff and showed clearly that oil accumulates rapidly during the early stages of seed development, after which further increase is comparatively slight. During that year, in collaboration with Morrell [1915], who drew attention to the commercial significance of this work, attempts were made to trace changes in the chemical characteristics of this oil as the seed developed and matured, but the difficulty of obtaining, at any particular stage of development or ripeness, sufficiently large and uniform quantities of seed was not overcome and the results, although of considerable interest, could only be regarded as tentative. The flax plant continues to flower day by day over a long period; the individual flowers last but a few hours; they are self-fertilised, after which, generally about noon, the petals fall and from then onwards the seed develops and ripens.

On resuming this work with Barker during 1921 and 1922 special precautions were taken to raise a perfectly uniform crop of flax and each season more than 8000 flowers were individually marked at stated intervals to provide the requisite quantity of seed for a fuller examination. The variety of flax grown in 1921 was that known commercially as Blue Flowering Dutch. Warm fine weather prevailed during the whole period of the trials. In 1922 two varieties were grown, namely the tall fibre-bearing blue-flowering *Linum usitatissimum* from Riga and *Linum cribrosum*, a short seed-bearing variety

from Madrid. Cool, generally wet, conditions prevailed and the season was fully two weeks later than in 1921. The plots were maintained in such good order as to ensure uniform development and ripening over the whole area.

In order to avoid oxidation all samples of seed collected were at once removed from the capsule and dried at 100° in a current of dry carbon dioxide, a procedure which avoided the possibility of after-ripening of the seed and the undesirable accumulation of water in the light petroleum used for the oil extraction. The following method was employed for determining the amount of oil present in the dried samples of seed taken from time to time. The weighed sample was ground with purified sand, transferred to a Soxhlet thimble and extracted with light petroleum (B.P. 50° to 70°) for 8 hours, taking care that the whole process was conducted in a current of dry carbon dioxide. The iodine values of these extracted oils were determined under the usual standard conditions by means of Wijs's solution, comparisons being made with a linseed oil of known iodine value.

In 1921 the first sample was picked 6 days after marking (flowering) and was found to contain only a trace of oil[1]. Subsequently 26 further samples were taken at short intervals over a period of 53 days after flowering. The seeds themselves when examined within the capsule at a very early stage of development appeared white and almost translucent. About the fifth day, as chlorophyll made its appearance in the young seeds, they assumed a greenish hue which increased to deep green by about the eighteenth or twentieth day. After this stage had been reached, when the seeds were fully plump and well grown, the green colour gradually disappeared and a browning set in, until, by about the fortieth day after flowering the seeds were a full brown colour, becoming darker as the seeds ripened. The analytical results of examining the seeds and the oil extracted therefrom are shown graphically as curves *A*, *B* and *C* in

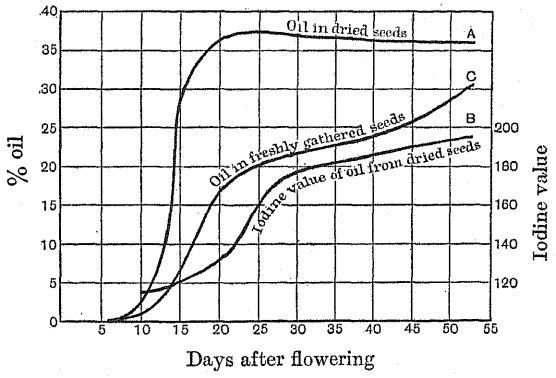


Fig. 1.

Fig. 1. On reference to these it will be seen that the synthesis of oleaginous extractable matter begins almost immediately after flowering and proceeds

[1] *I.e.* matter extractable by light petroleum.

comparatively slowly during the first 11 days. At the end of that time a rapid accumulation of oil sets in, amounting to about 3 % per day for some 10 days, when, after about 21 days from the time of flowering, the maximum oil content is reached and ceases with the disappearance of chlorophyll. As will be seen, observations were extended over a further period of 32 days, but during that period the variations in oil content were practically negligible when allowance is made for experimental errors due to sampling. Similar results were obtained with the two varieties grown in 1922, as will be seen on reference to the full line graphs shown in Fig. 2.

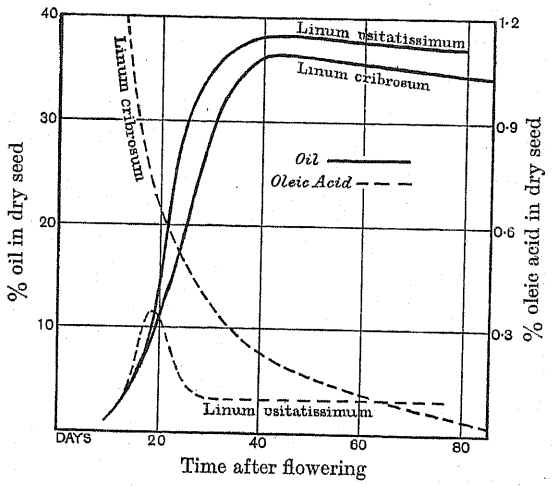


Fig. 2.

Changes in the character of linseed oil during seed development and ripening.

(i) *The colour of linseed oil.* During the course of this work wide variations were observed in the character of the linseed oil extracted at the various stages of development and ripeness of the seed. At first the oil was more than usually viscous and was of a greenish hue, but this gave place to a greater mobility and the characteristic full golden yellow colour as the age of the seed advanced. Further the oil became increasingly lighter in colour until, after 53 days from flowering, the seed yielded a very pale yellow oil and, when the harvested plants themselves, or merely the capsules containing the seeds, were stored under cover, indoors, the oil extracted from the seed was practically colourless.

(ii) *The unsaturated character of linseed oil.* In the case of a crop raised from *L. usitatissimum*, Dutch seed, the young seeds taken from the capsules when only 10 days old were found to yield an oleaginous residue having an iodine value of 114. Difficulties which attend the isolation of a sufficient quantity of this extract from seeds less than 10 days old prevent great reliance being placed upon analytical results obtained with such material, but it appears that the iodine value may well be as low as 100.

The iodine value of a good linseed oil varies between 180 and 200. On reference to Graph *B* in Fig. 1 it will be seen that the lower limit was reached after about 35 days, whilst the oil from seed 53 days old gave an iodine value of 191·9.

From a comparison of the graphs *A* and *B* in the same figure there appears to be no evidence of a change in the oil content parallel with the change in the iodine value of the oil. The interesting fact is revealed that the greatest rate of increase in the iodine value takes place after the oil content has attained its maximum value. The iodine value does not reach a maximum, but rises throughout the whole period during which observations were made, namely for 53 days after flowering, although the rate of increase diminishes somewhat towards the end of the period. The unsaturated character of the oil develops to a marked degree after the oil has been synthesised and the oil does not, as was generally supposed, become more and more saturated in character as the seed develops. Indeed, evidence points to the probability that changes in the degree of saturation are independent of those processes conditioning oil formation. Rapid oil formation occurs during those early days, 0 to about 20, when the colour both of the seed and of the extracted oil denotes the presence of chlorophyll; after that period, when the seed assumes a yellowish brown and full brown colour and the oil a full deep yellow colour, the iodine value of the oil rises rapidly, although no further increase in the oil content takes place. This view is supported by the following observed changes in the oil derived from seed when allowed to ripen under different conditions. It appears probable that these changes in the colour and iodine value of the oil are connected in some way either with residual life processes which persist in the plant, or are a separate set of changes controlled possibly by the carotene which persists throughout this period. Whether carotene is able to act so vigorously as an oxygen carrier as to remove hydrogen from the glyceride is not known, but from evidence available this does not seem improbable. The carotene itself may become oxidised during the final stages and thus account for the gradual loss of colour of the oil. In Table I will be found data relating to:

Material (*a*). Seed derived from detached capsules freshly gathered.

Material (*b*). Material as in (*a*) but set aside in the laboratory for varying periods.

Material (*c*). Seed derived from capsules not detached, but allowed to remain on the living plant while material (*b*) was after-ripening in the laboratory.

(iii) *The acidity of linseed oil.* The oils and fats which a plant produces from simple carbohydrates and stores as a reserve food material in its seed generally make their appearance as the seed approaches maturity, and, on germination, such reserve food is again converted into carbohydrates to support the young plant. In flax, however, it appears that oil formation sets in at an early stage, that the nature of the oil progressively changes, becoming more and more unsaturated in character, and contrary to expectations oil from fully mature seed is of greater value as a drying oil than is oil from less mature seed.

Table I.

Material (*a*) Seed from detached capsules freshly gathered			Material (*b*) Seed from detached capsules as (*a*) but after remaining at room temperatures for various periods						Material (*c*) Seed from capsules not detached from plant but allowed to remain on living plant while material (*b*) was at room temperatures				
Days after flowering	Oil %	Iodine value of oil	No. of days after (*a*)	Total days after flowering	Oil %	Increase of oil content %	Iodine value	Increase of iodine value	No. of days after flowering	Oil %	Increase of oil content %	Iodine value	Increase of iodine value
15	27·85	124·7	5	20	33·25	5·40	128·5	3·8	20	35·98	8·13	129·8	5
15	27·85	124·7	15	30	34·59	6·74	145·6	20·9	30	36·9	9·1	172	47
20	35·98	129·8	5	25	36·12	0·14	149·5	19·7	25	37·0	1·0	162	32
20	35·98	129·8	15	35	36·08	0·10	154·6	24·8	35	36·7	0·7	179	49
29	36·56	171·9	4	33	36·86	0·30	173·7	1·8	33	36·8	0·3	176	4

Glycerol, a substance closely related to the simple carbohydrates (sugars) from which presumably these compounds are formed, has been thought by some investigators to be the first product in the natural formation of linseed oil, the fatty acids being produced at a much later stage. Ivanoff, on the other hand, has suggested that in the synthesis of fats the higher saturated fatty acids are the first to be formed from the carbohydrates and that they are in turn converted into unsaturated acids which then combine with the glycerol present to form the fat.

It is of importance to have definite information on these points because for most technical purposes, as in the case of paint and varnish making, linseed oils of low acidity are highly esteemed. Should acid formation take place in the main during a definite period of seed development or ripening the character of the resulting oil may be in this respect influenced materially by harvesting the seed at a particular time. With the object of gaining information on these points in addition to following the course of oil formation, the acidity was measured of the oleaginous product obtained by extracting with light petroleum seeds of different degrees of ripeness. This was done by titrating with standard NaOH a solution of the oleaginous extract in neutral 90 % alcohol free from carbon dioxide, using phenolphthalein as indicator. The substances which are extracted from very young seeds by light petroleum are obtained as a solid or semi-solid mass when the solvent is evaporated. This extracted material is greenish-yellow in colour, readily becomes fluid when slightly warmed and is decidedly acidic.

On reference to Table II, columns 11, 12 and 13, it will be observed that as the seed of *L. usitatissimum* (Riga) ages the acidity of the oil diminishes rapidly, finally becoming almost constant at the low value of approximately 0·6. Commercial Baltic oil has an acidity of approximately 3 to 4 and Calcutta oil approximately 1 to 2. In the case of *L. cribrosum* (Madrid) similar results are recorded in Table III, columns 11, 12 and 13, from which it will be seen that in this variety of flax the oleaginous extract from the very young seeds is far more acidic than is the case with the *L. usitatissimum*, although it finally reaches a somewhat lower acid value. In this variety also the light petroleum extract from seeds 10 to 14 days old was obtained as a solid mass at ordinary

Table II. Linum usitatissimum (*Riga*).

	Freshly gathered seeds						Dry seeds			Oil from dry seeds		
				Calculated for 1000 seeds								
Age days	Wt. of 1000 seeds g.	Volatile at 100° %	Oil %	Vol. at 100° g.	Oil g.	Carbohydrates and protein g.	Wt. of 1000 seeds g.	Oil %	Colour	Acid value of oil*	Acid value (calculated) as oleic acid†	Remarks
1	2	3	4	5	6	7	8	9	10	11	12	13
12	6·3637	82·57	0·383	5·2545	0·0244	1·0848	1·1092	2·20	Greenish	—	—	Oil semi-solid at ordinary temperatures; oil fluid but of high viscosity; becoming less viscous as green colour disappears; oil much less viscous; marked decrease in viscosity and oil more readily oxidised
14	7·3264	80·88	0·553	5·9256	0·0405	1·3603	1·4008	2·89	yellow	8·6	43·428	
16	7·2771	78·17	1·207	5·6885	0·0878	1·5008	1·5886	5·53		8·4	41·454	
18	7·6685	76·89	2·078	5·8963	0·1594	1·6128	1·7722	8·99		7·8	38·493	
19	7·5838	74·63	2·935	5·6598	0·2226	1·7014	1·9240	11·57	Changing	4·8	24·083	
20	7·9179	74·92	4·015	5·9321	0·3179	1·6679	1·9858	16·01	gradually	4·2	20·727	
23	7·7178	68·89	7·267	5·3168	0·5609	1·8401	2·4010	23·36	from green	2·2	10·857	
25	7·6743	63·22	10·751	4·8517	0·8251	1·9975	2·8226	29·23	to deep	1·0	4·935	
27	7·5928	59·63	13·140	4·5276	0·9977	2·0675	3·0652	32·55	yellow	0·7	3·553	
30	7·7642	57·69	14·707	4·4688	1·1392	2·1382	3·2774	34·76		0·6	3·158	
32	8·1220	55·42	16·062	4·5012	1·3046	2·3162	3·6208	36·03	Green	0·6	2·961	
38	8·0422	47·92	20·254	3·8538	1·6289	2·5595	4·1884	38·89	disappeared	0·56	2·763	
46	7·6621	38·32	23·550	2·9361	1·7891	2·9369	4·7260	38·18	Pale yellow	0·60	2·961	
66	6·7209	28·70	26·673	1·9289	1·7927	2·9993	4·7920	37·41	Very pale	0·52	2·566	
76	6·3032	24·03	28·443	1·5147	1·7928	2·9957	4·7885	37·44	yellow	0·48	2·369	

Table III. Linum cribrosum (*Madrid*).

10	4·8426	84·43	0·11	4·0886	0·0053	0·7487	0·7540	0·68	Deep	—	—	Oil solid or semi-solid at ordinary temps.; oil fluid but of high viscosity becoming less viscous as green colour disappears
14	7·0844	81·76	0·56	5·7922	0·0397	1·2545	1·2922	3·08	greenish	83·0	420·462	
15	7·5610	79·77	0·83	6·0314	0·0628	1·4668	1·5296	4·09	yellow	—	—	
17	7·6892	77·35	1·46	5·9476	0·1123	1·6293	1·7416	6·45		23·3	118·045	
20	7·8856	75·88	2·44	5·9836	0·1924	1·7096	1·9020	10·11	More	14·2	72·248	
23	8·0137	72·19	4·62	5·7851	0·3702	1·8584	2·2286	16·62	intensely	7·0	35·532	
28	8·3667	67·71	8·97	5·6651	0·7505	1·9511	2·7016	27·79	green	3·2	16·582	
32	7·9594	60·60	13·07	4·8234	1·0403	2·0957	3·1360	33·18	Faintly	—	—	
36	7·6480	53·07	16·83	4·0588	1·2872	2·3020	3·5892	35·87	green	1·4	6·909	
44	8·6093	45·23	19·76	3·8940	1·7012	3·0141	4·7153	36·07	Green	1·1	5·525	Oil much less viscous; decrease in viscosity. Oil more readily oxidised
52	7·9823	41·16	21·14	3·2855	1·6875	3·0093	4·6968	35·93	absent	0·8	4·343	
66	7·3620	35·27	22·74	2·5966	1·6741	3·0913	4·7654	35·13	Very pale	0·7	3·553	
73	6·9107	31·90	24·01	2·2045	1·6593	3·0469	4·7062	35·26	yellow	0·18	0·928	
84	5·6634	16·81	29·32	0·9520	1·6605	3·0509	4·7114	35·24	oil	0·12	0·592	

* KOH per 1000 g. of oil. † Calculated as oleic acid per 1000 g. of oil.

temperatures. It is noteworthy also that this material was found to consist entirely of free acids, no indication being found of the presence of glycerides or other hydrolysable substances. It seems clear therefore that fatty acids are stored in the seed in the first instance, from which, presumably, the glycerides constituting the linseed oil are formed at a later stage. In Fig. 2 will be seen recorded graphically as full line curves the rate of development of the oleaginous extract or oil for the two varieties referred to, and as broken curves the corresponding acid development expressed in terms of oleic acid, as a convenient unit of acidity. The trend of these curves shows clearly how widely different the rates of acid formation in the seed of different varieties of flax may be, although the rate at which the oleaginous extract is stored in the seed is approximately the same in both varieties.

Through lack of material the lipase activity of the seeds at different stages of growth was not followed; it should certainly be done as this will probably throw interesting light on the mechanism of natural oil synthesis. If one may judge, however, from observations made regarding the activity of linase and the incidence of linamarin in the foliage and seeds of the same genus [Armstrong and Eyre, 1912] one may expect lipase activity to be coincident with glyceride formation.

Summary.

A study has been made of changes which take place in the rate of formation and in the character of the oil in the seed of flax—*Linum usitatissimum* and *L. cribrosum*—throughout its development and ripening period.

Observations show that in oil formation the acidic constituents are formed first. Whether the glycerol is formed at the same time, only combining with the fatty acid at a later stage, or whether the formation of glycerol itself is delayed is not clear. The observations reveal a remarkably rapid oil formation over a period of some 15 days, during which a maximum of about 36 % of oil, calculated on the dry weight of the seeds, is reached.

Changes in the nature of the oil continue after oil formation has ceased; its unsaturated character, as measured by its iodine absorption, continues to increase. It is pointed out that this is apparently an entirely separate change, independent of oil formation.

The interesting point is brought out that whereas during the early stages a reducing system must prevail in order to account for the formation of acids from carbohydrates, this must change to an oxidising system leading to the formation of unsaturated acids.

REFERENCES.

Armstrong and Eyre (1912). *Proc. Roy. Soc. Lond.* B **85**, 370.
Armstrong (1924). *J. Soc. Chem. Ind.* **43**, 207 T.
Eyre and Fisher (1915). *J. Agric. Sci.* **7**, 120.
Ivanoff (1912). *Beihefte Bot. Centr.* **28** (i), 159.
Morrell (1915). *J. Soc. Chem. Ind.* **34**, 109.

CCV. THE HYDROLYSIS OF CONCENTRATED SUGAR SOLUTIONS BY INVERTASE.

By HAROLD ALLDEN AUDEN and EDWARD ROMER DAWSON.

From the Research Department of the Distillers Company, Ltd., Epsom.

(*Received October 2nd, 1931.*)

Most of the extensive literature dealing with the kinetics of invertase action refers to the hydrolysis of dilute sucrose solutions. The commercial preparation of invert sugar from sucrose by hydrolysis in the presence of invertase necessitates the use of concentrated sugar syrups, and the object of the experiments reported was to study the relation between the rate of inversion and the sugar concentration.

Nelson and Schubert [1928] found that the velocity of hydrolysis by invertase decreased when the substrate concentration was greater than 10 %, the relation between sucrose concentration and velocity being approximately linear between 10 % and 70 % sucrose. This decrease in velocity was shown to be due to the falling off in water content.

Colin and Chaudun [1922] determined the relative viscosity of solutions containing from 10 to 60 g./100 cc. of sucrose and showed that the amount of sugar hydrolysed in a given time by a fixed amount of invertase decreased as the viscosity of the solution increased, the relation between fluidity and amount of hydrolysis being nearly linear. Since the viscosity of concentrated sucrose solutions is an exponential and not a linear function of the sugar content, it appears that the results of Colin and Chaudun are not in agreement with those of Nelson and Schubert.

The experiments of Colin and Chaudun were carried out at 23°, a temperature considerably lower than that of the critical inactivation of invertase. The optimum temperature for hydrolysis by invertase is generally taken to be 50–55° [*cf.* Oppenheimer and Kuhn, 1927; Waksman and Davison, 1926], but this value is not characteristic of the enzyme, being dependent on the time of the reaction. In view of the high negative temperature coefficient of viscosity, and the fact that an enzyme in the presence of its substrate is more resistant to high temperatures, it appeared probable that the apparent optimum temperature for the hydrolysis of concentrated sugar solutions by invertase would be higher than that for dilute substrate concentrations. The data obtained in the following experiments show that this is the case.

Experimental.

The invertase used was a very active preparation obtained from autolysed yeast. Its activity was tested from time to time and was found to remain constant during the period covered by the investigation. The stock enzyme solution was diluted immediately before use and the amount added to the substrate adjusted so that in all cases 100 cc. of sugar solution contained 0·4 cc. of invertase preparation. The stock enzyme solution was buffered with phosphoric acid to the extent that, when added in the above amount to sugar solutions, the resulting p_H was 4·4–4·6. No alteration in p_H was observed during the course of the hydrolysis.

To stop the hydrolysis 10 cc. of the reaction mixture were pipetted into a 100 cc. flask containing 80 cc. 0·25 % NaOH, and the solution was diluted with distilled water to 100 cc. at 15°. The mean delivery time of the pipette was taken as the time of observation. In view of the viscosity of the concentrated sugar solutions, the standard drain of the pipette was increased from 15 to 30 seconds, during which time the bulk of the sample was mixed with the alkali. The rotation of the sample was observed 15 to 30 minutes later. Readings were taken in a 400 mm. jacketed-tube round which water from a thermostat at 20° was circulated.

The amount of hydrolysis was calculated from the formula

$$\% \text{ hydrolysis} = \frac{R-d}{R-L}\,100,$$

where d is the observed rotation,
R is the initial rotation,
L is the rotation for complete inversion.

The initial rotation was determined for each concentration and temperature by adding to the sugar solutions the standard amount of invertase, the activity of which had been destroyed by diluting with NaOH. The final rotation was calculated from the relation

$$L = 0{\cdot}317\,R \text{ at } 20° \text{ [Hudson, 1910].}$$

The rotation of the invertase preparation was found to be negligible at the concentrations used.

The reaction velocity was calculated from the formula

$$k = \frac{1}{t}\log_{10}\frac{a}{a-x}.$$

In the experiments dealt with in Tables I–IV the amount of enzyme was constant, but the weight of substrate varied with the concentration. In order to calculate the activity of the enzyme in terms of the absolute weight of sugar hydrolysed in unit time, use was made of the expression:

$$\text{Invertase activity} = 10k \times \text{g. substrate.}$$

Table I. *The hydrolysis of solutions containing 70 g. sucrose/100 cc.*

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
45°	56	27·6	250	
	88	39·2	247	
	143	57·0	255	
	163	60·1	246	Average 250
50°	60	36·8	332	
	87	49·2	338	
	137	65·9	341	
	178	75·0	338	Average 337
57°	44	39·2	491	
	73	56·2	491	
	112	72·2	496	
	144	79·3	496	
	204	89·4	(478)	Average 493
62·2°	19·5	24·5	626	
	41·5	45·8	643	
	83	69·3	618	
	114	79·8	610	Average 624
65°	15	20·84	(677)	
	40	48·4	719	
	75	70·8	714	
	115	84·1	695	Average 709
68·8°	15	25·2	840	
	41·5	54·9	833	
	76·5	76·1	813	
	120	88·4	(780)	Average 829
72°	17·5	31·4	935	
	41·5	58·0	908	
	81	78·7	(830)	
	119	87·9	(771)	Average 921
75°	26·5	41·4	876	
	50	57·4	743	
	83	69·3	618	
	128	78·8	526	

Table II. *Hydrolysis of solutions containing 55 g. sucrose/100 cc.*

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
38°	54·7	35·2	344	
	91	53·5	365	
	128	66·4	370	
	163·7	75·6	374	
	225	86·0	379	Average 366
45°	25·2	25·1	(495)	
	60	51·7	527	
	88	66·5	540	
	117	76·7	541	
	165·5	86·5	526	Average 533
57°	30·7	47·0	898	
	56·3	70·2	933	
	80	81·7	922	
	107·5	89·2	898	Average 913
65°	20·5	44·3	1240	
	40	68·6	1260	
	71	87·8	1280	
	100	93·6	1190	Average 1240

Table III. *Hydrolysis of solution containing 40 g. sucrose/100 cc.*

T.	*t* (minutes)	% hydrolysis	$k \times 10^5$	
57°	18	49·5	1650	
	26·3	63·4	1660	
	25·7	75·0	1690	
	51·75	86·6	1680	Average 1680

Table IV. *Hydrolysis of solution containing 70 g. sucrose/100 g. solution (94·5 g./100 cc.).*

Digestion mixture 70 g. sucrose, 29·6 cc. water, 0·4 cc. invertase solution.

T.	*t* (minutes)	% hydrolysis	$k \times 10^5$	
57°	42	12·8	142	
	90·5	25·5	141	
	169	42·1	140·5	
	296	58·5	(130)	Average 141
65°	20	10·1	231	
	54	25·6	238	
	105	43·4	235	
	175	57·2	(211)	
	277	70·4	(191)	Average 235
70°	28·5	18·9	319	
	65·5	36·9	306	
	117·5	53·4	282	
	203	69·1	252	
	308	79·2	222	

The bracketed values for *k* were not used in estimating the mean value.

Table V.

T.	Concentration g./100 cc.	$10k$	$10k \times$ g. substrate
57°	40	0·167	6·68
	55	0·0913	5·02
	70	0·0493	3·45
	94·5	0·0141	0·99
65°	55	0·124	6·82
	70	0·0709	4·96
	94·5	0·0235	1·64

Fig. 1 shows that the relation between invertase activity and substrate concentration is linear.

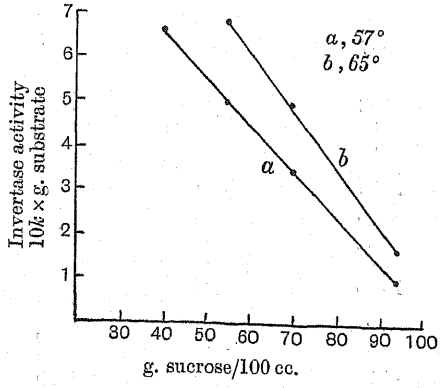


Fig. 1. Relation between invertase activity and substrate concentration.

The temperature coefficient.

From the results given in Table I it appears that for a digestion period of a few hours the apparent optimum temperature for the hydrolysis of 70 % sucrose in the presence of invertase is about 70°.

Experiments on the hydrolysis of 10 % sucrose by invertase at the same p_H showed that at 65° the value for k decreased very rapidly, while at 68° the hydrolysis ceased after 20 minutes owing to the inactivation of the enzyme.

The temperature coefficient of the reaction is also greater for concentrated, than that found by Nelson and Bloomfield [1924] for dilute, solutions. Calculation of the values of the critical increment in calories per gram-molecule gave the following results:

Table VI.

$$E = R\frac{T_1 - T_2}{T_2 - T_1}\log_e\frac{k_2}{k_1}.$$

Conc. of sugar	$T°$ (abs.)	$k \times 10^5$	Temp. range ° C.	Mean temp. $t°$ C.	E (found)	E (calc.)
70 %	318	250	45 –50	47·5	12,300	12,500
	323	337	45 –57	51	11,900	11,900
	330	493	50 –57	53·5	11,600	11,500
	335·2	624	57 –65	61	10,100	10,200
	338	709	57 –68·8	62·9	9,900	9,900
	341·8	829	62·2–72	67·1	9,200	9,200
	345	921	65·2–72	68·6	8,900	9,000
55 %	311	366	38 –45	41·5	10,600	10,600
	318	533	45 –57	51	9,400	9,600
	330	913	57 –65	61	8,500	8,500
	338	1240	—	—	—	—
94·5 %	330	141	57 –65	61	14,200	—
	338	235	—	—	—	—

The experimental values for E are plotted against the mean temperature in Fig. 2 and are in agreement with the equations

$$E = 20{,}500 - 168t \text{ for 70 \% sucrose,}$$
$$E = 15{,}100 - 108t \text{ for 55 \% ,,}$$

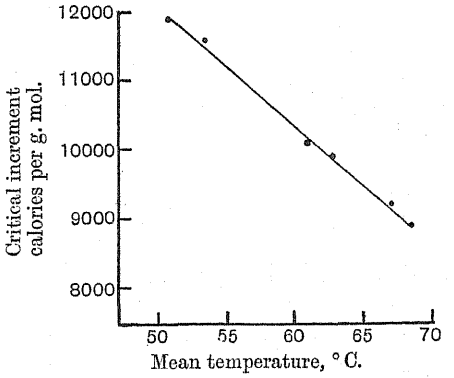


Fig. 2. Variation of the critical increment with temperature. Sucrose concentration 70 g./100 cc.

Nelson and Bloomfield [1924] combined the data in the literature for the hydrolysis of dilute sugar solution by invertase and found that the observed values could be calculated from the equation

$$E = 12{,}300 - 117t.$$

In order to ensure that the divergence between the values for E for the different substrate concentrations was not due to the enzyme preparation, the rate of hydrolysis of 10 % sucrose by invertase at p_H 4·6 was determined at 38° and 56·5°. The values of k for the unimolecular reaction were found to increase regularly as the hydrolysis proceeded and a different method was used to calculate the relative rates of hydrolysis at the two temperatures. The time t_z required by the reaction mixture to reach zero rotation (75·9 % hydrolysis) was found to be 61 and 32 minutes at 38° and 56·5° respectively. Using the reciprocals of these values to denote the relative velocities, the value of E was calculated to be 7100: the corresponding figure calculated from the equation of Nelson and Bloomfield for a mean temperature of 47·5° was 6800.

Viscosity and rate of hydrolysis.

In order to test the statement of Colin and Chaudun that the amount of hydrolysis by invertase of concentrated sucrose solutions is proportional to the fluidity of the solution, the viscosities of the solutions used were calculated. For this purpose Orth's equation [Browne, 1912] relating the viscosity of concentrated sugar solutions to the temperature and concentration, was used:

$$\log_e(\log_e\eta) = \log_e(\log_e A) + x\log_e B + t\log_e C,$$

where η is the viscosity,

x is the concentration in g./100 g. solution,

t is the centigrade temperature, and

A, B and C are constants.

Applying viscosity data from Browne [1912] and from Landolt-Börnstein [1923] the equation was simplified to the following:

$$\log_e(\log_e\eta) = 3{\cdot}619 + 0{\cdot}07798x - 0{\cdot}02260t.$$

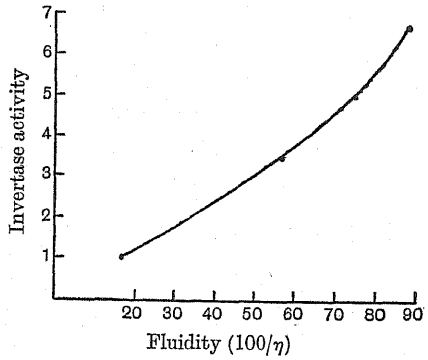


Fig. 3. Relation between the fluidity of sugar solutions and invertase activity. Temperature 57°.

In Fig. 3 the fluidity ($100/\eta$) of sucrose solutions at 57° is plotted against the invertase activity (Table VI) for the corresponding concentration. The linear relationship found by Colin and Chaudun is not observed.

Discussion.

According to Euler and Laurin [1919] the critical inactivation temperature for invertase is 59°. The observations described above lead to the conclusion that invertase resists exposure to much higher temperatures when in the presence of a large excess of substrate. The increased rate of hydrolysis observed at temperatures above that of critical inactivation is not merely an increase in the initial velocity, since the observations covered a large portion of the range of inversion. In this connection it should be noted that the course of the hydrolysis by invertase of 70 % sucrose follows that of a unimolecular reaction much more closely than does the hydrolysis of 10 % sucrose, although deviations from the mass law might be expected with the more concentrated solutions.

The larger values of the temperature coefficient of the hydrolysis of concentrated, compared with that for dilute, solutions cannot be ascribed to the increased rate of hydrolysis due to decrease in viscosity with rise in temperature, since this would not account for the greater resistance to temperature of the enzyme in presence of 70 % sugar. A concentration of 10 % sucrose is greater than that required to produce the maximum initial velocity of hydrolysis by invertase [*cf.* Kuhn, 1923], and presumably the enzyme is already saturated with substrate at this concentration. By applying the conception of Willstätter that an enzyme consists of an active substance stabilised by the presence of a colloidal carrier, the resistance of the enzyme-complex to temperature in the presence of sugar syrup may be explained as due to the increased stability of the carrier in this medium.

In Figs. 1 and 3 it is shown that the decrease in the rate of hydrolysis with increasing sucrose concentration is more closely related to the decrease in water content than to the increase in viscosity. This is in agreement with the results of Ingersoll [1926] who found that the initial velocity of sucrose hydrolysis by invertase at 25° did not vary with the increase in viscosity due to increase in sugar concentration in the manner described by Colin and Chaudun. Ingersoll also showed that the initial velocity was not decreased by the addition of gelatin in amounts sufficient to produce a considerable increase in viscosity. It may be concluded, therefore, that the viscosity of the medium is not the factor responsible for the observed variations in the catalytic activity of invertase.

The values calculated for the critical increment are subject to a possible correction for the variation of the optimum p_H with temperature and substrate concentration. Nelson and Bloomfield, however, have shown that the small shift in optimum p_H with temperature is towards the alkaline side. This means that the actual estimations were carried out slightly on the acid side of the

optimum. Since Nelson and Bloomfield have shown that the value of the critical increment is smaller at acidities greater than the optimum, it follows that any correction would increase the value of E for the concentrated solutions, and so increase the difference between the values for the dilute and concentrated substrate.

SUMMARY.

1. The rate of hydrolysis by invertase of solutions containing 40 % or more of sucrose decreases as the substrate concentration increases, the relation between substrate concentration and invertase activity being linear.

2. The optimum temperature for the hydrolysis of 70 % sucrose in the presence of invertase is 65–70°.

3. The relation between the critical increment in calories per gram-molecule and the centigrade temperature for the hydrolysis of 70 % sucrose by invertase can be expressed by the relation

$$E = 20{,}500 - 168t.$$

REFERENCES.

Browne (1912). Handbook of sugar analysis, p. 310. (Chapman and Hall, London.)
Colin and Chaudun (1922). *Bull. Soc. Chim. Biol.* **4**, 272.
Euler and Laurin (1919). *Z. physiol. Chem.* **108**, 64.
Hudson (1910). *J. Ind. Eng. Chem.* **2**, 143.
Ingersoll (1926). *Bull. Soc. Chim. Biol.* **8**, 264.
Kuhn (1923). *Z. physiol. Chem.* **125**, 28.
Landolt-Börnstein (1923). Physikalisch-Chemische Tabellen, **1**, 139. (Springer, Berlin.)
Nelson and Bloomfield (1924). *J. Amer. Chem. Soc.* **46**, 1025.
—— and Schubert (1928). *J. Amer. Chem. Soc.* **50**, 2188.
Oppenheimer and Kuhn (1927). Lehrbuch der Enzyme, p. 257. (Thieme, Leipzig.)
Waksman and Davison (1926). Enzymes, p. 179. (Baillière, Tindall and Cox, London.)

CCVI. SYNTHESIS OF *i*-β-HYDROXYGLUTAMIC ACID.

By CHARLES ROBERT HARINGTON
and SYDNEY STEWART RANDALL.

WITH A NOTE ON THE DISSOCIATION CONSTANTS.

By George Maxwell Richardson.

From the Department of Pathological Chemistry, University College Hospital Medical School, and the Biochemistry Department, Imperial College of Science and Technology, South Kensington.

(*Received October 9th, 1931.*)

The synthesis of β-hydroxyglutamic acid described by Dakin [1919] leaves much to be desired in the matter of the yields obtainable. Since the isolation of this amino-acid from natural sources also offers very considerable difficulties, the compound has not been available in sufficient amount to permit of an extended examination of its chemical and physiological properties.

According to Dakin [1918] β-hydroxyglutamic acid occurs in caseinogen to the extent of about 10 %. In view therefore of its importance as a constituent of protein and still more owing to the recent claim of Dakin, West and Howe [1930] to have isolated from liver a compound, active in curing pernicious anaemia, which yields on hydrolysis one molecule of hydroxyproline and one of β-hydroxyglutamic acid, the latter compound is of considerable biological interest and it was thought to be desirable to re-investigate the possibilities of its synthesis by a more practicable method.

Among the numerous possible syntheses explored by Dakin [1919] the simplest and most attractive from the theoretical point of view consisted in the reduction of the ethyl α-*iso*nitrosoacetonedicarboxylate of Pechmann [1891] which is an easily accessible substance. Dakin's own attempt to effect this reduction, using sodium amalgam under a variety of conditions met with failure, owing probably, as he himself suggests, to secondary reactions on the part of the aminoketo-ester which would be formed during the first stage of the reduction; nor could any more satisfactory results be obtained by the reduction of ethyl α-*iso*nitroso-β-ethoxyglutaconate.

It occurred to us that catalytic methods of reduction might find an application in this problem. It is true that the catalytic reduction of the *iso*nitroso- to the amino-group and still more that of the carbonyl to the secondary alcoholic group have, until recently, been regarded as difficult of execution. The activating effect of small amounts of iron on the catalytic reduction of carbonyl com-

pounds observed by Carothers and Adams [1923] and by Faillebin [1923] has, however, largely removed the difficulties in this case, and it has further been shown by Hartung and Munch [1929] that the reduction of an *iso*nitroso-compound to the corresponding primary amine is favoured by the presence of excess of hydrogen chloride in the reaction mixture. The latter authors have indeed achieved the direct reduction of several α-*iso*nitrosoketones of mixed aliphatic-aromatic type to the corresponding amino-alcohols, the reaction being carried out in alcoholic solution containing three molecular equivalents of hydrogen chloride and the catalyst employed being palladinised charcoal. It is to be noted that Hartung *et al.* [1930] met with exceptional difficulties in the case of *iso*nitrosoacetophenone, which was readily reduced to the amino-ketone but could be reduced to the alcohol only after substitution of water for alcohol as the solvent and introduction of fresh catalyst.

When the reduction of ethyl α-*iso*nitrosoacetonedicarboxylate was attempted by the method of Hartung and Munch [1929] it was found that hydrogen was rapidly absorbed up to 2 mols., *i.e.* in amount corresponding with the reduction of the ester to ethyl α-aminoacetonedicarboxylate. That the latter was indeed the product of the reaction at the stage under discussion was proved as follows: when boiled with 20 % hydrochloric acid it lost alcohol and carbon dioxide and the residue, when treated with alkali and mercuric chloride and steam-distilled, gave a satisfactory yield of 2 : 5-dimethylpyrazine; furthermore the partial reduction product gave, when heated with *p*-nitrophenylhydrazine in excess of dilute sulphuric acid, the characteristic *p*-nitrophenylosazone of methylglyoxal.

The reduction could not be carried beyond this stage at a reasonable rate under the conditions already described. If, however, the solution were evaporated and the residue re-dissolved in water, the remaining stage of the reduction could be accomplished in presence of the original catalyst activated by the addition of chloroplatinic acid and a trace of iron. Even so, the second stage (reduction of the carbonyl group) was much slower than the first (reduction of the *iso*nitroso-group). When the uptake of hydrogen finally ceased the primary amino-nitrogen in the solution represented only about 40 % of the total nitrogen. It was apparent therefore that some ring closure had occurred, probably to give a pyrrolidonecarboxylic acid derivative; in any case the whole of the nitrogen could be converted into the amino-condition by boiling with 20 % hydrochloric acid, a process which simultaneously removed the ester groups.

At this point in the earlier experiments hydrochloric acid was removed as far as possible by evaporation of the solution under diminished pressure and the amino-acid was isolated from the residue through its silver salt as described by Dakin [1918]. Subsequently, however, the favourable properties of the hydrochloride (see below) have been utilised to obtain the bulk of the product directly as a crystalline derivative. In either case the total yield of the amino-acid amounted to about 60 % of the theoretical calculated on the ethyl α-*iso*nitrosoacetonedicarboxylate employed.

The free β-hydroxyglutamic acid obtained in the earlier experiments, being finally isolated by precipitation of its concentrated aqueous solution with alcohol, formed an amorphous powder apparently similar to the product obtained by Dakin. On one occasion, however, the mother-liquor, which contained more water than usual, deposited a little of the amino-acid after long standing in the form of crystalline crusts, and since that time no difficulty has been experienced in obtaining the acid in a crystalline condition from concentrated aqueous solution.

Since β-hydroxyglutamic acid contains two independent asymmetric carbon atoms it is evident that the synthetic product may consist of either one of two racemates or of a mixture. We have at present no satisfactory evidence to offer as a basis for a decision between these alternatives. Although we have at times observed the separation of the acid in crystals of apparently different forms, these forms have not shown any marked differences of solubility, nor have we been able to standardise the conditions of their separation. The more usual type of crystals obtained consists of stout prisms containing water of crystallisation which show no tendency to separate into different components when fractionally crystallised.

The constitution of the synthetic acid was proved by analysis of the acid itself, of its hydrochloride and of various metallic salts; also by the formation of malic semialdehyde as the result of oxidising the acid with 1 mol. of chloramine-*T*. Unfortunately we have so far been unable to isolate the amino-acid from natural sources, so that no direct comparison between the natural and synthetic products has been possible. It is indeed unlikely that any such comparison would be useful for purposes of identification, since for stereochemical reasons the physical properties of the naturally occurring active acid may well be profoundly different from those of the synthetic material. That some such marked difference does indeed exist is indicated by certain contrasts between the behaviour of our product and that of the amino-acid isolated by Dakin.

i-β-Hydroxyglutamic acid crystallises from concentrated aqueous solutions in well-formed prisms containing three molecules of water of crystallisation; it is extremely soluble in hot water but only moderately so in cold. Under certain circumstances, *e.g.* crystallisation from very concentrated solutions at a high temperature, the acid may separate in the anhydrous condition. The hydrated acid melts at 75°, re-solidifies and finally decomposes at 185°; the anhydrous compound melts sharply with decomposition at 198°. This is in marked contrast with the behaviour described by Dakin [1918] whose product had no sharp melting-point, but even at 105° lost water with ring closure to a hydroxypyrrolidonecarboxylic acid. No such ring closure takes place when the dry synthetic acid is heated at 110° even in a vacuum over phosphoric oxide. On the other hand, ring closure does occur when the amino-acid is boiled in aqueous solution, and at p_H 4 the process is considerably more rapid than with glutamic acid under similar conditions.

The synthetic amino-acid yields a well-formed hydrochloride somewhat

similar in character to glutamic acid hydrochloride although naturally more soluble than the latter. No mention of such a derivative is made by Dakin either in connection with natural or synthetic β-hydroxyglutamic acid. We now suspect, however, from some observations which we have made ourselves on the character of the crude precipitate of glutamic acid hydrochloride obtained from caseinogen in the usual manner, that unsuspected losses as the hydrochloride may account in part at least for the many failures to isolate β-hydroxyglutamic acid. In this connection the claim of Engeland [1922, footnote p. 136], based on methylation experiments, that a commercial preparation of glutamic acid hydrochloride contained as much as 50 % of β-hydroxyglutamic acid is of interest.

Finally as to colour reactions. Dakin described two principal colour reactions as characteristic for β-hydroxyglutamic acid, (*a*) a red colour with sodium diazobenzenesulphonate in presence of excess of sodium hydroxide, which deepened in intensity on warming, and (*b*) a green fluorescence on warming a solution of the acid with excess of sulphuric acid and a trace of β-naphthol. The pure synthetic amino-acid gives no colour whatever with sodium diazobenzenesulphonate and sodium hydroxide in the cold; on boiling the solution a bluish-red colour with no trace of yellow tinge is obtained[1]. The reaction is not, however, very sensitive. The green fluorescence with β-naphthol and sulphuric acid is given by the synthetic amino-acid but again not with great intensity. In any case we do not regard either of these reactions as of great value. Traces of histidine entirely obscure the result of the former test, and there appear to be substances present in protein hydrolysates which give the β-naphthol-sulphuric acid reaction with even greater intensity than β-hydroxyglutamic acid itself.

It appears therefore that marked discrepancies exist between the behaviour of our synthetic product and that of the acid isolated by Dakin from natural sources. Many of these discrepancies could doubtless be explained on stereochemical grounds and it is therefore better not to discuss them further until the resolution of the synthetic acid has been effected and until a reliable method for the isolation of the natural acid has been devised. The first of these problems we hope to take up in the near future; with the second we have been occupied for some time without success. We have repeatedly followed with meticulous care the procedure recommended by Dakin [1918] but have invariably failed to obtain the acid in the manner which he describes. The fraction which should contain β-hydroxyglutamic acid has always consisted of a small amount of gummy material more or less heavily contaminated with glutamic and aspartic acids. That β-hydroxyglutamic acid does indeed occur among the products of hydrolysis of caseinogen we do not for a moment doubt; unequivocal proof of this is afforded by the fact that certain fractions of the

[1] In a private communication to one of us Dr H. D. Dakin agrees that, contrary to his original statement, no colour should be given with sodium diazobenzenesulphonate and sodium hydroxide in the cold.

hydrolytic products yield not inconsiderable amounts of malic semialdehyde when oxidised with chloramine-*T*. We are, however, forced to the conclusion that the separation of β-hydroxyglutamic acid from the other dicarboxylic amino-acids is not by any means so straightforward a process as the original description by Dakin [1918] might lead one to suppose. The mere separation of a synthetic mixture of glutamic and β-hydroxyglutamic acids into its components is a task of considerable difficulty, although our experiments on this point, detailed description of which is reserved for a later communication, have afforded indications of possible lines of attack on the major problem. In the meantime the synthesis of the inactive amino-acid and its properties together with those of some of its simple derivatives are described below.

One of us (C.R.H.) has, during the progress of this work, enjoyed the benefit of consultation by correspondence with Dr H. D. Dakin, concerning the various points which have arisen.

Experimental.

Preparation of ethyl α-isonitrosoacetonedicarboxylate.

The following method has been found more convenient for the preparation of the above compound in quantity than that described by Pechmann [1891].

Ethyl acetonedicarboxylate (20 g.) is dissolved in ether (200 cc.) in a 500 cc. round-bottom flask under a reflux condenser. Hydrogen chloride is passed into the solution at the rate of 2–3 bubbles per second. A solution of ethyl nitrite [*cf.* Thiele and Eichwede, 1900, footnote p. 366] (8·4 cc.) in ether (50 cc.) is added in 10 cc. portions through the condenser. Each addition of ethyl nitrite causes a yellowish-brown coloration and a slight rise of temperature; a fresh quantity of ethyl nitrite is only added when the colour produced by the preceding addition has been discharged. After the final addition of ethyl nitrite, passage of hydrogen chloride is continued for 15 minutes, and the mixture is then set aside overnight. Next day the bulk of the ether is distilled off, and the residue is twice shaken out with excess of dilute sodium carbonate solution. The latter is then acidified with hydrochloric acid and extracted four times with ether. The combined ethereal extracts are dried over calcium chloride and evaporated, finally in a vacuum desiccator over sulphuric acid. The yield of pure ester is almost quantitative.

Reduction of ethyl α-isonitrosoacetonedicarboxylate.

The catalyst was prepared by dissolving 1 g. of palladium chloride in 100 cc. of water with the addition of 1 cc. of concentrated hydrochloric acid, adding 6 g. of "super-norite" charcoal and shaking in an atmosphere of hydrogen until reduction was complete. The product was filtered off, washed with water until free from chloride and dried in a vacuum desiccator. Ethyl α-*iso*nitrosoacetonedicarboxylate (10 g.) was dissolved in absolute alcohol (100 cc.) containing hydrogen chloride (5 g.); 3 g. of the palladium-charcoal were added and

the mixture was shaken in an atmosphere of hydrogen. Absorption of the latter occurred rapidly and continued at a moderate rate until 2 mols. had been taken up when the process became extremely slow; in various experiments this first stage occupied 1·5 to 2·5 hours.

When the absorption had practically ceased the catalyst was filtered off and washed with alcohol; the filtrate and washings were freed from alcohol by evaporation under diminished pressure and the residue was taken up in water (100 cc.). The original catalyst was returned to the solution and in addition there were added 5 cc. of a solution containing 10 % of chloroplatinic acid and 0·5 % of ferric chloride. Hydrogenation was then begun again and allowed to proceed until the remaining 1 mol. had been absorbed. When this had occurred (after 6–8 hours) the solution was filtered. The catalyst was extracted with boiling water and the combined filtrates were evaporated to 100 cc. under diminished pressure.

i-β-Hydroxyglutamic acid.

The solution obtained as described above should theoretically contain ethyl β-hydroxyglutamate. It was found, however, that only about 40 % of the total nitrogen was in the amino condition. Concentrated hydrochloric acid (100 cc.) was therefore added and the whole was boiled under a reflux for 1·5 hours. After this treatment the amino-nitrogen and total nitrogen were identical. Subsequent treatment followed two alternative lines.

(*a*) The solution was evaporated under diminished pressure, the process being repeated several times after addition of small amounts of water. The residue was taken up in water and treated with 20 % silver nitrate in amount just sufficient to precipitate the remaining hydrochloric acid. The filtrate from the silver chloride was made faintly alkaline to litmus and further silver nitrate solution was cautiously added. The precipitate produced by the first few drops immediately blackened, owing doubtless to the persistence of some reducing material. The addition of silver nitrate solution was carefully continued until a transient white precipitate was produced; at this stage the solution was set aside overnight. Next day the black precipitate was removed by filtration and the amino-acid was completely precipitated from the filtrate by alternate addition of 20 % silver nitrate and 2*N* sodium hydroxide. The silver salt was filtered off, thoroughly washed with water, and decomposed with hydrogen sulphide. The filtrate from the silver sulphide was concentrated to a thin syrup under diminished pressure and poured into excess of alcohol. The precipitate, at first sticky, gradually hardened and was ultimately obtained as a friable powder; it was dried in a vacuum desiccator over phosphoric oxide.

(*b*) After the properties of the well-characterised hydrochloride had been discovered it was found more convenient to evaporate the hydrochloric acid solution resulting from hydrolysis of the reduction product to a thin syrup under diminished pressure and to saturate the residue at 0° with hydrogen

chloride. After standing overnight in the ice-chest the hydrochloride was filtered off and the remainder of the amino-acid was isolated from the mother-liquor by the method described above. The free amino-acid was obtained in the crystalline condition by addition of 1 equivalent of sodium hydroxide to the concentrated aqueous solution of the hydrochloride, or by dissolving the amorphous preparation obtained as described above in a small amount of hot water and allowing the solution to stand. Occasionally, if the solution be very concentrated and warm when crystallisation commences, the acid separates in the anhydrous condition in stout needles or narrow rhombic prisms. More usually, however, it is obtained as stout prisms which may reach a considerable size and contain $3H_2O$; the latter is lost in a vacuum over phosphoric oxide.

Analysis. 0·1554 g. of the air-dried acid lost 0·0378 g. *in vacuo* at 50° over phosphoric oxide. Found: loss of weight 24·3 %; calculated for $C_5H_9O_5N$, $3H_2O$, loss of weight 24·9 %.

The anhydrous acid gave: C, 36·5, 36·8 %; H, 5·6, 5·5 %; N, 8·5, 8·6 %; amino-N, 8·7 %. $C_5H_9O_5N$ requires: C, 36·8 %; H, 5·5 %; N, 8·6 %.

0·2082 g. required for neutralisation to phenolphthalein 12·48 cc. of 0·099 *N* sodium hydroxide. The acid therefore titrates as a monobasic acid of mol. wt. 168 as against the calculated value of 163.

Behaviour on heating. When heated in a capillary tube the air-dried acid melts partially at about 75° and then re-solidifies; at 102–103° obvious loss of water occurs and the whole finally decomposes at 185°. The anhydrous acid melts sharply with vigorous effervescence at 198°. No apparent change occurs when the dry amino-acid is heated at 110° either in the air or in a vacuum over phosphoric oxide. That ring closure can be brought about with moderate ease is shown, however, by the following experiment. 0·5 g. of the acid was dissolved in 5 cc. of water and the solution was boiled under reflux for 7 hours, by which time the ninhydrin reaction had become very faint. Analysis indicated more than 80 % loss of amino-nitrogen. The solution was evaporated to a syrup on the water-bath and the residue was extracted with alcohol. The extract was filtered from unchanged amino-acid and evaporated again to a syrup which slowly crystallised. The product had N, 9·4 % (calculated for $C_5H_7O_4N$; N, 9·65 %) and yielded only a trace of nitrogen in the Van Slyke apparatus. A comparison between the rates of ring closure of glutamic and β-hydroxyglutamic acids was kindly carried out by Prof. A. C. Chibnall who obtained the following figures (the acids were boiled in aqueous solution at p_H 4).

	% of total N as amino-N after		
	1 hr.	2 hrs.	3 hrs.
Glutamic acid	96·9	93·0	84·3
β-Hydroxyglutamic acid	81·2	75·5	62·7

Reactions. The acid, after neutralisation to litmus, gives an intense ninhydrin reaction. With sodium diazobenzenesulphonate and excess of sodium hydroxide it gives no colour in the cold; on boiling a bluish-red colour develops.

When an aqueous solution of the acid is treated with rather more than its own volume of sulphuric acid and the mixture is warmed with a trace of β-naphthol a yellowish solution with a green fluorescence results. The vapours evolved on fusing the acid with zinc dust give an intense pine splinter reaction for pyrrole.

Oxidation with chloramine-T. 0·815 g. of the acid in 5 cc. of water was neutralised with *N* sodium hydroxide (5 cc.) and treated with a solution of chloramine-*T* (1·44 g.) in water (5 cc.). Almost immediate precipitation of toluenesulphonamide occurred. After 30 minutes the reaction was completed by gentle warming, the solution was chilled and the toluenesulphonamide filtered off. The filtrate was treated with a hot solution of *p*-nitrophenylhydrazine (2 g.) in glacial acetic acid and transferred to the steam-bath. Precipitation of the osazone began at once; after 45 minutes the precipitate was filtered off, washed with warm alcohol and dried; it weighed 0·5 g. After recrystallisation from nitrobenzene it formed brownish-red prismatic needles, M.P. 291° (uncorr.). (Found: N, 22·6 %. $C_{16}H_{14}O_6N_6$ requires: N, 22·6 %.)

Derivatives of i-*β-hydroxyglutamic acid.*

Hydrochloride. This was prepared as described above. It forms colourless prismatic needles, readily soluble in water, sparingly so in concentrated hydrochloric acid. It has M.P. 187° (decomp.). (Found: C, 29·8, 30·0 %; H, 5·0, 5·1 %; N, 7·1, 7·0 %; amino-N, 7·3 %; Cl, 17·7 %. $C_5H_{10}O_5NCl$ requires: C, 30·0 %; H, 5·0 %; N, 7·0 %; Cl, 17·8 %.)

Ethyl ester hydrochloride. β-Hydroxyglutamic acid hydrochloride (7 g.) was suspended in alcohol (70 cc.) and the mixture was saturated with hydrogen chloride. The resulting clear solution was boiled under reflux for 3 hours and then concentrated under diminished pressure. During this process the greater part of the ester hydrochloride crystallised out. This was filtered off (6 g.) and recrystallised from alcohol. M.P. 168·5°. (Found: N, 5·2 %. $C_9H_{18}O_5NCl$ requires: N, 5·5 %.)

Silver salt. Prepared by adding silver nitrate (2 mols.) to a solution of the disodium salt of β-hydroxyglutamic acid (1 mol.) filtering, washing and drying in a vacuum desiccator in the dark. (Found: N, 3·6 %. $C_5H_7O_5NAg_2$ requires: N, 3·7 %.)

The normal *copper* salt had N, 5·9 %, Cu, 28·0 % (calc.: N, 6·2 %, Cu, 28·3 %) and the *calcium hydrogen* salt had Ca, 11·5 % (calc.: Ca, 11·0 %). These salts were prepared according to Dakin [1918] and resembled the products described by him.

Identification of intermediate product in the reduction of ethyl α-iso*nitrosoacetonedicarboxylate.*

(*a*) Ethyl α-*iso*nitrosoacetonedicarboxylate (5 g.) was subjected to the first stage of the reduction in the manner described above. The residue obtained by evaporation of the alcoholic solution was dissolved in 20 % hydrochloric acid (50 cc.) and boiled under reflux. Decarboxylation set in rapidly and was

complete in 45 minutes. The solution was evaporated under diminished pressure, the evaporation being several times repeated after addition of small amounts of water. The residue was taken up in water (35 cc.) and the solution was added with cooling to 80 cc. of 33 % potassium hydroxide; 10 g. of mercuric chloride dissolved in cold water (100 cc.) were then added and the whole was immediately steam-distilled. After 75 cc. had passed over the base had practically ceased distilling. The distillate was treated with a solution of mercuric chloride which produced a crystalline precipitate of the mercuric chloride double salt with 2 : 5-dimethylpyrazine; after standing overnight the precipitate was collected, washed and dried in a desiccator. It amounted to 5 g. corresponding with a yield of 70·5 % of the theoretical calculated on the ester originally taken. (Found: Hg, 62·2 %. $C_6H_8N_2$,2HgCl requires: Hg, 61·7 %.) The base recovered from the mercuric chloride salt was further identified by conversion into the *picrate*, M.P. 157°. (Found: picric acid, 68·5 %; calculated, 67·9 %), and the *chloroaurate*, M.P. 153°.

(*b*) The residue from the evaporation of the alcoholic solution of the first stage reduction product from a second quantity of 5 g. of ethyl α-*iso*nitrosoacetonedicarboxylate was treated with a solution of *p*-nitrophenylhydrazine (5 g.) in excess of hot 5 % sulphuric acid and transferred to the steam-bath. Within a few minutes the solution became filled with the characteristic scarlet needles of methylglyoxal *p*-nitrophenylosazone. After 1 hour the precipitate was filtered off, washed with alcohol and dried (1·4 g.). After recrystallisation from nitrobenzene it had M.P. 297° (decomp. uncorr.).

NOTE ON THE DISSOCIATION CONSTANTS.

By GEORGE MAXWELL RICHARDSON[1].

Application of amino-acid dissociation constants to the interpretation of protein titration curves is a recognised procedure which depends upon accurate knowledge of these constants and concise statement of the conditions of measurement. Data are now presented for crystalline preparations of β-hydroxyglutamic acid, accessible to measurement for the first time.

Bjerrum's zwitterion hypothesis, which possesses the cumulative experimental evidence of Borsook and MacFadyen [1930], Michaelis and Mizutani [1925], and Birch and Harris [1930, 1, 2] in support of its more logical interpretation of the behaviour of amino-acid solutions, is adopted throughout the text. Basic dissociation constants are calculated as constants of hydrion dissociation, after Brønsted [1923]. These generally accepted conventions,

[1] Beit Scientific Research Fellow.

presented more fully by Greenstein [1931], allow an abbreviated formulation of the acid-base exchanges as follows:

$$\overset{-\,H^+}{{}^+A} \rightleftharpoons \overset{-\,H^+}{{}^+A^-} \rightleftharpoons \overset{-\,H^+}{{}^+A^{--}} \rightleftharpoons A^{---}.$$

Decreasing magnitudes, K_1', K_2', K_3', represent the successive dissociation constants (uncorrected for activity of the various species of A) possible within this scheme. In this paper, however, calculation of the slightly different

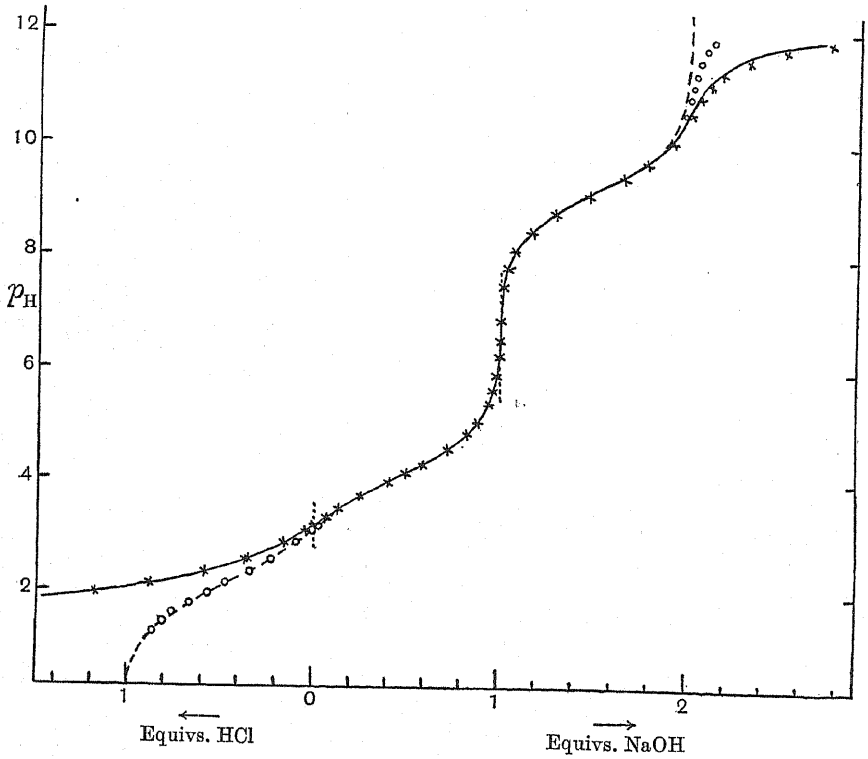


Fig. 1. Titration of 0·02 *M* β-hydroxyglutamic acid, 25°.

Calculated titre, uncorrected ———— Corrected - - - - - - -
Experimental titre, uncorrected × Corrected o

titration constants of Simms [1926], G_1', G_2', G_3', is made. It is to be emphasised that electrometric titration of any dicarboxylic amino-acid is attended by the usual difficulties of technique and calculation relating to comparatively acid solutions, and that this susceptibility to error will necessarily appear in the values of G_1'.

Experimental data are presented in Fig. 1, the curve representing calculated requirements, the points experimental findings. Table I presents a portion of these data arranged for calculating Simms's titration constants. Experimental titres are corrected throughout for the concentration of uncombined HCl or

NaOH at the stated p_H. Bracketed values represent determinations in poorly buffered regions, or values embodying heavy correction. The Sørensen logarithmic nomenclature (p_{G_1}, *etc.*) is adopted.

Table I. *Calculation of titration constants.*

25°. 0·02 *M* β-hydroxyglutamic acid, $3H_2O$. 0·1012 *M* NaOH. 0·1972 *M* HCl.

p_{G_2}' (0·1122 g. acid in 25 cc.)

NaOH cc.	p_H	μ	Correction (equivs.) for H^+	Correction (equivs.) for $+A^-$	Correction (equivs.) for $+A^{--}$	α_2	p_{G_2}'
0	3·219	0·00193	0·0299	0·0704	—	0·1003	4·172
0·32	3·368	0·00262	0·0216	0·0510	—	0·1352	4·174
0·65	3·518	0·00347	0·0156	0·0366	—	0·1794	4·178
1·25	3·753	0·00531	0·0093	0·0216	—	0·2756	4·173
2·02	4·019	0·00782	0·0053	0·0119	—	0·4126	4·172
2·50	4·176	0·00936	0·0037	0·0083	—	0·5014	4·174
3·00	4·337	0·0109	0·0027	0·0057	—	0·5957	4·169
3·70	4·598	0·0131	0·0015	0·0032	—	0·7291	4·168
4·24	4·863	0·0147	0·0008	0·0017	—	0·8326	4·166
4·51	5·054	0·0155	0·0005	0·0011	0·0001	0·8845	4·170
4·81	5·381	0·0161	0·0003	0·0005	0·0002	0·9423	{4·168
5·01	5·900	0·0167	0·0001	0·0002	0·0005	0·9806	4·196
5·06	6·240	0·0168	—	0·0001	0·0011	0·9896	4·262}
5·108 (= first equiv.)							Mean 4·172

p_{G_1}' (0·1089 g. in 25 cc.)

HCl cc.	p_H	p_{G_1}'
0	3·221	2·10
0·125	3·106	2·09
0·40	2·886	2·09
0·90	2·595	2·10
0·93	2·582	2·10
1·49	2·357	2·09
2·25	2·149	2·11
3·01	1·989	{2·11
4·25	1·803	2·10
6·01	1·634	2·14
9·76	1·418	2·06
13·60	1·299	2·10}
		Mean 2·10

p_{G_3}' (0·1122 g. in 25 cc.)

NaOH cc.	p_H	p_{G_3}'
5·12	6·881	9·20
5·20	7·485	9·20
5·30	7·817	9·23
5·49	8·126	9·22
5·92	8·474	9·21
6·60	8·801	9·20
7·49	9·119	9·20
8·41	9·423	9·19
9·10	9·686	9·18
9·75	10·057	9·19
10·25	10·553	{9·20
10·51	10·860	9·19}
11·10	11·262	—
11·76	11·497	—
12·77	11·700	—
14·02	11·859	—
		Mean 9·20

In discussion of the data various points arise. Firstly, the table provides an interesting comparison between the very constant p_{G_2}', values, where ionic strength and H^+ concentration are both comparatively low, and the erratic p_{G_1}', or the decreasing p_{G_3}' values, where a large correction for free H^+, or a rapidly increasing ionic strength, respectively, are involved. Secondly, certain evidence for a fourth (hydroxy) dissociation constant at about p_H 13 may be detected in Fig. 1, though the absence of any satisfactory basis for correcting for free OH^- precludes definite evaluation of the constant. An attempt to eliminate the effect by larger OH^- correction disturbs the terminal p_{G_3}' values and fails to give good alignment of the end-point. Thirdly, preliminary titration of a preparation, dried at 50° over P_2O_5, revealed that titratable

groups about p_H 9 exceeded the more acid titratable groups by about 4 %. A further recrystallisation, however, reduced the extent of the divergence to about 2 %, approaching the limits of possible experimental error. The interfering $p_{G'}$ lay between 9·0 and 9·4. As neither analysis nor method of preparation offers an easy explanation for a defect of —COOH as against $-NH_2$ groups, the observation is merely recorded. Fourthly, it may be noted that an air-dried sample of the β-hydroxyglutamic acid containing $3H_2O$ was found to titrate with respect to its acid groups as 75 % hydroxyglutamic acid by weight (theory 75·1 %). The anhydrous preparation titrated as 99 % acid.

Table II. *Dissociation constants at 25°.*

	Mol. conc.	—COOH	—COOH	$-NH_2$	Diff. $(p_{K_{NH_2}'} - p_{K_{NH_2}'})$	Authors
Glutamic acid	—	2·15	4·22	9·60	—	Various [*vide* Kirk and Schmidt, 1929]
β-Hydroxyglutamic acid	—	2·32	4·23	9·56	0·04	[Kirk and Schmidt, 1929]
β-Hydroxyglutamic acid	0·02	2·09	4·18	9·20	0·40	This paper
β-Asparagine	0·02	2·02	—	8·80	—	[Chibnall and Cannan, 1930]
β-Hydroxyasparagine	0·01	2·12	—	8·26	0·54	" " "
Aspartic acid	0·02	2·05	3·76	9·85	—	" " "
Hydroxyaspartic acid	0·02	1·95	3·47	9·03	0·82	" " "

The comparison of the constants here determined with those of Kirk and Schmidt [1929] reveals definite discrepancy in the case of the third (amino) dissociation constant. It is believed, however, that the synthetic origin and purity of our preparation, and the satisfactory mathematical analysis accorded by the data are sufficient evidence for the greater reliability of the present constants. This belief is further supported by the comparisons of Table II, in which our data are compared with those of Kirk and Schmidt, and with the data of Chibnall and Cannan [1930] for hydroxyaspartic acid and its amides. The influence of an adjacent hydroxyl group on the $p_{G'}$ of the amino-group is to be noted. In particular, the glutamic acids should be compared with the β-asparagines, where there is likewise an α-amino group, a β-hydroxy group, and an almost non-polar γ-group.

Finally, for general reference, the $p_{G'}$ constants are converted to the real dissociation constants by the formula of Simms [1926]. Thus, the successive values of $p_{K'}$, determined by titration of 0·02 *M* β-hydroxyglutamic acid at 25°, become 2·09, 4·18 and 9·20.

Experimental.

Titrations were conducted in the manner of Cannan and Knight [1927]. Reagents and apparatus were prepared and standardised with the usual precautions. Temperature was maintained closely at 25°. The half-cell adopted was a saturated calomel electrode, standardised against 0·1 *M* HCl from a constant boiling-point stock. Junction was made at the tip of a goose-neck saturated KCl bridge of the Clark type. The H_2 electrode potential assumed for 0·1 *M* HCl was 0·0637v., junction potentials being deliberately ignored throughout.

Care was observed particularly during acid standardisation or titration, to prevent fortuitous junction potentials arising from a dead space in the tip, containing, between the KCl and the external liquid, a solution of some previous p_H. On the other hand, increase of ionic strength of the solution due to KCl introduction was minimised. Potentials were established at duplicate, freshly palladinised, gold-plated platinum wire electrodes, and measured against a Cambridge potentiometer and galvanometer. In no observation did duplicate potentials differ by more than 0·2 mv. and there was generally agreement closer than 0·1 mv. An exception arose, however, with those less pure preparations already mentioned, variations up to 0·4 mv. occurring about p_H 10.

Finally, due mention must be made of the basis upon which observed H^+ activities (p_H) were converted into H^+ or OH^- concentrations. The simplified Debye-Hückel equation, $-\log\gamma = a\sqrt{\mu}$, where a varies with the dielectric constant of the solution and the degree of solvation of the ions, was used. In these calculations, a has been accepted, empirically, as a constant, 0·245, for both H^+ and OH^-.[1]

In calculation of μ, the ionic strength, it is assumed for simplicity that a positive and negative charge on the same ion are mutually neutralised in their influence on the ionic atmosphere. Thus $^+A^-$ is disregarded and $^+A^{--}$ is regarded as a univalent ion [compare Greenstein, 1931]. On this basis, μ increases most rapidly during the reaction $^+A^{--} \rightarrow A^{--}$, which would produce just that gradual decrease in $p_{G_3'}$ values which Table I reveals. On the other hand, the full assumption is contrary to the conclusions of Borsook and MacFadyen [1930], who wittingly offer no alternative formulation for the modified participation of such ions. On the stated basis, μ is readily shown to equal the sum

$$[^+A] + [H^+] + [Na^+] + [A^{--}],$$

where, with negligible error,

$$[^+A] = [A]\frac{H^2}{(G_1+H)(G_2+H)}, \text{ and } [A^{--}] = [A]\frac{G_3}{(G_3+H)},$$

[A] representing total amino-acid concentration and H, H^+ activity. OH^- activities were derived from H^+ activities by assuming Lewis, Brighton, and Sebastian's value [1917] for p_{K_w}, 13·995, without modification for altered dielectric constant.

[1] The activity of these two ions in solutions of moderate ionic strength is shown by data of Lewis and Randall to be closely comparable, while the above value of a is of similar order to that adopted by Simms for H^+ (0·28). It differs from the decreasing value of Randall and Breckenridge [1927] for chloride solutions of increasing ionic strength. It was found that the values of these authors gave no concordant data in the calculation of $p_{G_1'}$. The critical survey of Borsook and MacFadyen [1930] is sufficient justification in this case for selecting an empirical constant.

REFERENCES.

Birch and Harris (1930, 1). *Biochem. J.* **24**, 564.
—— —— (1930, 2). *Biochem. J.* **24**, 1080.
Borsook and MacFadyen (1930). *J. Gen. Physiol.* **13**, 509.
Brønsted (1923). *Rec. Trav. Chim.* **42**, 718.
Cannan and Knight (1927). *Biochem. J.* **21**, 1384.
Carothers and Adams (1923). *J. Amer. Chem. Soc.* **45**, 1071.
Chibnall and Cannan (1930). *Biochem. J.* **24**, 945.
Dakin (1918). *Biochem. J.* **12**, 290.
—— (1919). *Biochem. J.* **13**, 398.
—— West and Howe (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Engeland (1922). *Z. physiol. Chem.* **120**, 130.
Faillebin (1923). *Compt. Rend. Acad. Sci.* **177**, 1118.
Greenstein (1931). *J. Biol. Chem.* **93**, 479.
Hartung and Munch (1929). *J. Amer. Chem. Soc.* **51**, 2262.
—— Munch, Deckert and Crossley (1930). *J. Amer. Chem. Soc.* **52**, 3317.
Kirk and Schmidt (1929). *Univ. California Publ. Physiol.* **7**, 57; *J. Biol. Chem.* **81**, 237.
Lewis, Brighton and Sebastian (1917). *J. Amer. Chem. Soc.* **39**, 2245.
Michaelis and Mizutani (1925). *Z. physikal. Chem.* **116**, 135.
Pechmann (1891). *Ber. deutsch. chem. Ges.* **24**, 860.
Randall and Breckenridge (1927). *J. Amer. Chem. Soc.* **49**, 1435.
Simms (1926). *J. Amer. Chem. Soc.* **48**, 1239.
Thiele and Eichwede (1900). *Liebig's Ann.* **311**, 363.

CCVII. STUDY OF THE BLOOD-CALCIUM FOLLOWING THE INTRAVENOUS INJECTION OF CALCIUM SALTS.

By GUY DRUMMOND GREVILLE.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

(*Received October 10th, 1931.*)

THROUGH the intravenous administration of calcium salts the blood suffers only a transitory increase in calcium concentration, for the blood-calcium sinks rapidly, returning usually within 2 hours of the injection to its original level. This has been observed by several workers in different animals, notably by Heubner and Rona [1919, 1] in cats, by Hetényi [1924] and Gerschmann [1930] in dogs, and by Clark [1920], Condorelli [1926, 1927] and Rothlin [1930] in rabbits. The last two authors and Taylor and Fine [1930] have commented on the remarkably large apparent loss of calcium from the blood-stream during the first 5 minutes after the injection. The object of the experiments described below was firstly to discover whether this apparent loss could be attributed in any measure to shortcomings of the analytical method, and secondly to study the kinetics of the fall of the blood-calcium.

Material.

In addition to calcium chloride, there was used in this work calcium levulinate, a very soluble calcium salt described by Conrad [1878], the value of which as an agent for calcium therapy by injection has recently been pointed out [Dodds and Greville, 1931; Bennett, 1931]. Pure calcium levulinate was kindly supplied by the Crookes Laboratories.

Adequacy of the analytical method.

In most of the researches on the behaviour of the serum-calcium following calcium administration, the calcium has been determined by direct precipitation as oxalate from diluted serum, usually by the technique of Kramer and Tisdall [1921] as modified by Clark and Collip [1925]. This much-used procedure of direct precipitation has recently been severely criticised. Incomplete calcium precipitation due to the presence of serum-proteins tends to lower the result [Van Slyke and Sendroy, 1929; Guillaumin, 1930], while at the same time entrainment of oxidisable foreign matter with the oxalate precipitate tends to raise it. Guillaumin [1930] concluded that with fresh human blood the second error preponderates. So that although direct precipitation has often been shown in animal serum to give the same results as

ashing [Loucks and Scott, 1929–30; Hendriks, 1929], it must be recognised that error may possibly accompany its use.

Experimental.

It was first shown that quite good recoveries were obtained by the Clark and Collip technique when large quantities of calcium were added to serum. To 2 cc. of horse serum in the precipitation-tubes measured volumes of standard calcium chloride solution were added. Analyses were performed in quadruplicate, the extreme error being less than 2 %. Recoveries of 98·4 % and 97·6 % were obtained with 0·577 mg. and 0·289 mg. of added calcium respectively.

The above results were obtained when serum and dilute calcium chloride solution were mixed in the precipitation-tube. If, on the other hand, a mixture was made by adding, with thorough shaking, 1 cc. of a stronger calcium solution to 49 cc. of serum in a graduated flask, 2 cc. portions of this mixture gave, when analysed by direct precipitation, low and erratic results. Thus with a mixture made as above from horse serum and calcium levulinate solution, with a total calcium content of 39·0 mg./100 cc., values ranging from 10 to 33 mg./100 cc. were obtained. Similar results were obtained with a mixture of calcium chloride solution and horse serum. The error was not due to insufficient mixing since consistent, if rather high, recoveries were obtained when the calcium was determined in the mixture following precipitation of the proteins by trichloroacetic acid. No systematic alteration occurred when determinations were made after leaving the mixture for many hours and centrifuging, or when the oxalate precipitation was allowed to proceed for 24 hours. The supernatant liquid after centrifuging the precipitate, and the washings, were observed to be cloudy; hence under these conditions the calcium oxalate is precipitated in a readily peptisable form, and variable amounts of colloidal calcium oxalate are decanted off in the supernatant fluid. It is interesting to note that Grollman [1927] found that, when calcium chloride was added to serum *in vitro* in amounts (up to a total concentration of 32 mg./100 cc.) which caused no precipitation or change in concentration of inorganic phosphate, the latter was rendered increasingly non-diffusible.

When strong calcium solutions are injected into the veins of living animals, the addition of calcium to serum takes place under circumstances similar to those in which the erratic results described above were obtained. It was therefore considered desirable to compare the serum-calcium value as determined by direct precipitation with that obtained by some reliable fundamental method, in blood abstracted from a living animal a short time after an intravenous injection of a strong calcium solution had been made. This experiment was of further interest in that Heubner [1925] found that after the injection into cats of calcium lactate, as well as of calcium hexosephosphate, only a part of the calcium could be precipitated directly as oxalate.

Accordingly (Exp. 1, Table I) a rabbit was put under open ether anaesthesia,

70 mg. of calcium as calcium levulinate in 5 cc. of solution were injected into the external jugular vein, and 5 minutes later about 30 cc. of blood were obtained rapidly from the carotid artery. In the heparin plasma obtained from the blood, the calcium was determined both by the Clark and Collip method, and also by the wet combustion method recently described by Widmark and Vahlquist [1931]. (The use of the filter-stick was replaced by the usual Clark and Collip technique, after precipitation in the cold and standing overnight.)

It will be seen (Table I) that the Clark and Collip method gave a value for the plasma-calcium 2·2 % higher than that obtained by the incineration method. We may therefore conclude that under these conditions the direct precipitation method is competent to give an accurate measurement of the plasma-calcium.

Table I. *Exp. 1.*

Weight of rabbit = 2·80 kg. Ether anaesthesia.
Injection = 70 mg. of calcium as calcium levulinate in 5 cc. solution.
Blood taken 5 mins. after injection.
Haematocrit reading: (mean of 4) 30 % corpuscles.
Plasma-calcium (Clark and Collip technique):
27·8, 27·5, 27·4; mean 27·6 mg./100 cc.
Plasma-calcium (Widmark and Vahlquist ashing technique):
27·0, 27·1, 26·9; mean 27·0 mg./100 cc.
Whole blood-calcium (Widmark and Vahlquist ashing technique):
20·0, 19·6, 19·1; mean 19·6 mg./100 cc.
70 % × 27·0 = 18·9 mg./100 cc.
Difference = 0·7 mg./100 cc.

(Blank tests: 0·0056 mg. Ca per cc. nitric acid: no allowance necessary for other reagents.)

Two further ways in which calcium in the blood may escape determination by the serum-calcium analysis may be envisaged: it may pass into the corpuscles, or it may be present as a suspension of insoluble calcium salt which would be carried down with the corpuscles. To discover whether either of these events occurred, in the above experiment the calcium content of the whole blood was determined by the Widmark and Vahlquist [1931] method and haematocrit readings were taken. It will be seen (Table I) that the calcium in whole blood is greater than the amount calculated from the ashed plasma value by 0·7 mg./100 cc. or 3·6 %. This may well be within the limits of experimental error. Heubner and Rona [1919, 1] found quantities of calcium in the corpuscles of calcium-injected animals varying from 0 to 7 mg./100 cc.; but it may be mentioned that even with normal blood there is still controversy as to the calcium content of the corpuscles, two of the latest determinations giving zero [Leiboff, 1930] and varying quantities up to 2 mg./100 cc. [Guillaumin, 1930].

From Exp. 2 (Table II) more data may be obtained. Here the whole blood-calcium was 0·7 mg./100 cc. lower than the value calculated from the plasma content. The latter value however was determined by direct precipitation which we have seen gives slightly higher results. At any rate we may conclude from

Table II. *Exp. 2.*

Weight of rabbit = 3·12 kg. Ether anaesthesia.
Injection as in Exp. 1, zero time to 0 min. 30 secs.
Bleeding, 1 min. 15 secs. to 3 mins. 15 secs.
Mean haematocrit reading = 73 %.
Blood-calcium (ashing technique):
21·9, 22·0, 22·2; mean 22·0 mg./100 cc.
Plasma-calcium (Clark and Collip):
15 mins. after drawing blood, 31·0, 31·0 mg./100 cc.
3 hrs. 30 mins. after drawing blood, 31·0, 31·4 mg./100 cc.
73 % of 31·1 mg./100 cc. = 22·7 mg./100 cc.

these experiments that the quantity of calcium which under the circumstances described is carried down with or inside the corpuscles on centrifuging is negligible.

Lastly, although we have no evidence of precipitation *in vivo* from the above experiments, it seemed desirable to discover whether calcium precipitated from the blood on standing *in vitro* at room temperature. Although Greenberg and Gunther [1930] had shown that with normal ox serum the serum-calcium value obtained is independent of the time (up to the 22 hours studied) during which the serum stands in contact with the clot, it did not follow that the serum of our experiments, so heavily charged with calcium, would be equally stable. The blood was heparinised, a portion rapidly centrifuged and the plasma-calcium determined as soon as possible. A second portion was kept, loosely corked, for 3½ hours, after which it was centrifuged at high speed for 15 minutes, and the plasma-calcium again determined. The value was found to be no lower, and to agree with the whole blood value on the assumption that there was no calcium in the corpuscles.

From the above experiments it may be concluded that the serum-calcium value as determined by the direct precipitation method gives an accurate measure of the blood-calcium after the intravenous injection of calcium levulinate.

Study of disappearance of calcium from the blood-stream.

Control experiments. In order satisfactorily to construct a curve showing the fall of serum-calcium after the injection, it was necessary to make sure that the serum-calcium returned finally to a constant level. In the curves constructed by previous authors, the serum-calcium is usually still falling when it reaches the initial level again. Accordingly attention was directed towards finding conditions under which, in the absence of injection, but with the degree of haemorrhage necessary for the withdrawal of the blood samples, the serum-calcium would stay constant for a length of time equal to the duration of the experiment.

Now the serum-calcium of the rabbit (which animal was used for these experiments as it was the most convenient) is by no means as constant as that of man or of other animals such as dogs. Thus Derevici [1929] gives the

serum-calcium of rabbits as 12–20 mg./100 cc. Most notable is the raising of the serum-calcium of the fasting rabbit by the ingestion of cabbage [Culhane, 1927, 1930]. Although this was disputed by Kapsinow and Underhill [1929], the writer has found a systematic difference between the serum-calcium levels of rabbits which have been continuously fed on cabbage and those of rabbits which have not. Thus the values for 23 cabbage-fed rabbits ranged from 14·7 to 16·9 (average 15·6), while the values for 18 rabbits of the second group, which had been fed on oats or on bran or which had taken no food since the previous evening, ranged from 8·2 to 14·9 (average 11·6) mg./100 cc. There is less variation in the cabbage-fed group. Rabbits kept continuously on this diet have a steady blood-calcium, as was shown by Davies, Dickens and Dodds [1926]. For the present purpose it was therefore decided to keep cabbage continuously in the cage both during the experiment and for at least 3 hours before. With these conditions, control experiments involving bleedings at the times to be used in the succeeding experiments showed a satisfactory steadiness of the serum-calcium, as in Exp. 3 (Table III).

Table III. *Exp. 3.*

Rabbit, female, 2·50 kg.

Time ...	0	5	20	40	60	120	180 mins.
Serum-calcium	15·0	14·6	14·5	14·2	14·1	14·4	14·5 mg./100 cc.

Technique.

The injection was made into the peripheral vein of the left ear. A puncture was made with a needle of triangular section in the peripheral vein of the right ear, and the blood allowed to flow into a centrifuge-tube where it clotted; the serum-calcium was then determined by the Clark and Collip technique. 5 cc. of blood were usually obtained in less than a minute, but in the later stages of the experiments a longer time was often consumed. The mid-time of the bleeding was noted. In these experiments no blood was taken before 5 minutes after the injection, as owing to the rapid fall of blood-calcium at this stage, the comparatively long times taken over the bleeding would introduce large inaccuracies.

Table IV. *Exp. 4.*

Rabbit, female, 3·32 kg. Dose = 13·6 mg. Ca in 1 cc. per kg., as calcium levulinate.

Time ...	(Initial)	0	5	20	43	65	125	270 mins.
Serum-calcium	14·7	(Injection)	22·9	19·6	17·0	15·9	14·9	15·1 mg./100 cc.
Excess above final level	—	—	7·9	4·6	2·0	0·9	—	— ,,
Log_e (excess)	—	—	2·07	1·53	0·69	−0·11	—	—
Antilog *A*	—	—	9·5	9·5	9·4	9·3	—	—

Table V. *Values of antilog* A.

Blood sample no. ...	1	2	3	4	5
Exp. 4	9·5	9·5	9·4	9·3	—
,, 5	11·2	10·2	13·8	10·5	—
,, 6	10·7	10·7	9·6	10·5	11·0
,, 7	10·7	10·2	10·7	10·0	—

Table VI.

Exp. no.	Weight (kg.) and sex	Dose (mg. Ca per kg.) and salt	k	Approximate time to reach constant level (mins.)	Antilog A
4	3·32 (F)	13·6 (L)	0·037	110	9·5
5	3·18 (M)	16·0 (L)	0·036	120	11·2
6	3·18 (M)	13·6 (L)	0·021	210	10·3
7	3·36 (M)	14·5 (C)	0·037	150	10·6
8	3·04 (M)	16·0 (L)	(0·086)	40	(8·5)
9	3·10 (M)	14·5 (C)	(0·047)	80	(9·1)
10 Cat*	2·28 (M)	16·0 (L)	0·023	140	7·6

L = Calcium levulinate. C = Calcium chloride. * Under chloralose anaesthesia.

Table VII.

Exp. 11. Rabbit 3·0 kg. Injection 13·1 mg. Ca per kg. as 10 % levulinate.
Inorganic phosphate* in (citrated) plasma:
Before injection 4·0 mg. P/100 cc.
5–6 mins. after injection 4·7 ,,

Exp. 12. Rabbit 3·04 kg. Injection as in Exp. 11.
Inorganic phosphate in serum:
Before injection 4·5 mg. P/100 cc.
4–5 mins. after injection 4·9 ,,

* de Wesselow [1924].

Table VIII.

Exp. 13. Rabbit, 3·03 kg. Injection 31 mg. Ca per kg. as calcium chloride.
Haematocrit reading: 75 % plasma.

	Serum-calcium*	Haemoglobin†	Alkali reserve‡
Before injection	14·2	100 units	29
3–6 mins. after injection	32·0	92·6 units	25
Increase	17·8	−7·4 %	−4
	mg./100 cc.		cc. CO_2/100 cc.

4 cc. CO_2 at N.T.P. ≡ 3·6 mg. Ca

* Clark and Collip [1925]. † Myers [1924]. ‡ Van Slyke [1917].

Results.

The results are included in Tables IV–VI. A typical curve is shown in Fig. 1, in which the serum-calcium values found in Exp. 4 are plotted against

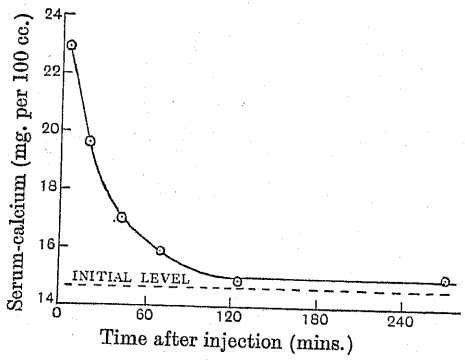


Fig. 1.

the time after the injection. The curve is concave upwards, thus differing from the curves given by Hetényi [1924], but agreeing with those of Gersch-

mann [1930] and Rothlin [1930]. It will be observed that the final steady level differs little from the initial level: this was not so in all the experiments, the final level being sometimes higher and sometimes lower than the initial level. The greatest observed difference between the two was, however, only 1·4 mg./100 cc. There was one experiment (not included in the Tables) in which the serum-calcium did not return to a constant level, but fell steadily after reaching the initial value. In all the experiments doses of the same order of magnitude were given.

Discussion.

It is of interest to see whether there is any approximate relation between the excess of the serum-calcium at any instant above the final constant level and the time to which it corresponds. In the third row of Table IV the values

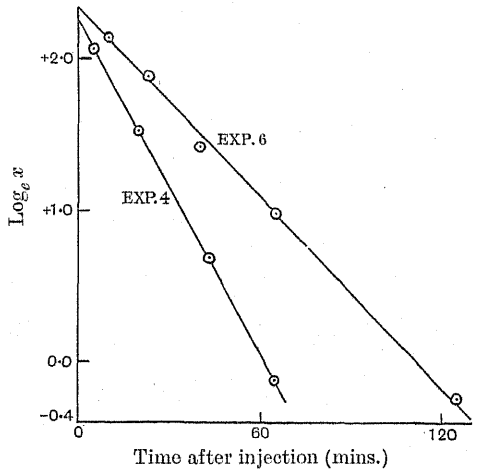


Fig. 2.

of this excess (x) are given for each point of Exp. 4. In Fig. 2 $\log_e x$ is plotted against the time after the injection (t), and it will be seen that the points lie on a straight line. x and t are therefore connected by the relation

$$\log_e x = A - kt,$$

where k and A are constants for the particular experiment. Hence the rate of fall of calcium excess at any instant is directly proportional to the excess obtaining at that instant. A is the logarithm of the (theoretical) initial value of the excess, while k is the rate of fall per unit calcium excess (for Exp. 4, 0·037 mg./100 cc. per minute per mg./100 cc. excess).

In order to determine how closely the data fit this logarithmic law, we may insert in the above equation the values for x, t and k, and so calculate A for each point of the curve. From the constancy of A we may draw conclusions concerning the accuracy with which the law is obeyed. The corresponding values of antilog A for the points of Exp. 4 are given in the fifth

row of Table IV, and the constants for significant analyses in other rabbit experiments are given in Table V.

Out of the experiments performed, three satisfactory curves were obtained with calcium levulinate and one with calcium chloride. In addition there were some experiments in which the rate of fall of serum-calcium was so rapid that enough points on the significant part of the curve were not obtained to give logarithmic graphs of interest (data given in brackets in Table VI, in which the results of the important experiments are summarised).

It must be remembered, in interpreting the results obtained, that plotting data on a logarithmic scale tends to smooth out divergences; but an examination of Fig. 2 and Table V shows that the data are distributed evenly about the curve representing the logarithmic law, and do not show any systematically increasing divergence. It would of course be idle to suggest that the relationship is exactly accurate or to attach any physical significance to it, for we are dealing with the net result of several complicated processes and the return to a dynamic equilibrium. We can, however, say that the data are conveniently represented by this equation, and that the rate of disappearance of serum-calcium at any instant is approximately proportional to the excess above the steady value which obtains at that instant. Such a formulation has indeed previously been employed in the kinetics of blood chemistry: for example Fishberg [1930] found that the disappearance of foreign sugars from the blood-stream can be described by this (to use a chemical analogy) "unimolecular law."

It will be seen from Table VI, from the values of k and of the approximate time taken for the serum-calcium to return to a constant level, that the rate at which calcium disappears from the blood-stream varies greatly among the different animals. Exp. 7 with chloride yields results which fall in line with those with levulinate [*cf.* Rothlin, 1930]; there might well have been a difference since calcium chloride is said to disturb the acid-base equilibrium of the blood.

Interest attaches to the constant "antilog A." This initial highest value of the serum-calcium is never of course realised in practice owing to disappearance of calcium from the blood-stream during the injection, and also because the injected calcium takes some time to become completely mixed with the circulating blood [*v.* Erlanger, 1921]. But it gives an approximation to the amount of serum-calcium which would disappear during the course of the experiment if the fall of serum-calcium during the first 5 minutes were to exhibit the same relationships as it does during the later part of the experiment. It is found that the loss calculated for the whole experiment is quite inadequate to account for the total disappearance. Thus in Exp. 4, 13·6 mg. of calcium per kg. are injected. Now from the table given by Erlanger [1921] it appears that 50 cc. is a reasonable figure to take for the blood-volume per kg. of rabbit, and as 70 % is a usual haematocrit reading for rabbit's blood we may suppose that there are 35 cc. of serum per kg. The potential increase

in serum-calcium is therefore $\frac{13{\cdot}6 \times 100}{35} = 39$ mg./100 cc. But antilog A is only 9·5. Hence about 30 mg./100 cc. have not been accounted for. Even if we take the improbable figure of 9 % for the blood-volume, the unexplained disappearance amounts to 12 mg./100 cc., a quantity greater than the total loss calculated by extrapolation from the curve. Obviously, in addition to the disappearance of serum-calcium at a rate expressed approximately by the logarithmic relationship, there is a rapid disappearance immediately after the injection. This idea is borne out by a curve given by Rothlin [1930]; following injection of 29 mg. Ca per kg. the rate of fall during the first 20 minutes is relatively very much greater than that during the succeeding 100 minutes. It is reasonable to suppose that two different processes take place.

The analyses of Heubner and Rona [1919, 2; 1923], Jungmann and Samter [1924], Hecht [1924] and Heubner [1925] showed that, after toxic doses, the only significant rise in the calcium content of the tissues occurred in the kidneys. This, together with the calcium in urine and intestinal contents, did not account, a few hours after the injection, for the large disappearance of injected calcium from the blood-stream: deposition of calcium in the bones seemed the only explanation. Hetényi [1924], in an animal experiment, recovered after 3 hours only 15·6 % of the injected calcium from urine and faeces. In the same animal, before and after injecting a lethal dose (294 mg. per kg.) of calcium, he obtained a 2 % increase in the calcium concentration of the bone, which may be significant.

Dadlez [1926] found that the urinary calcium excretion was abnormally high at the end of 6 hours after injection. Brull [1930] on the other hand [*cf.* also Proverman and Brull, 1930] considers that the renal calcium excretion following intravenous calcium chloride injection is dependent entirely on the rate of flow of urine, thereby differing from Hetényi and Nógrádi [1925], who found in normal human beings a trebled calcium concentration in the urine during the first 30 minutes after injection. Recently Taylor and Fine [1930], by washing out loops of isolated intestine [*cf.* Stewart and Percival, 1927], found that the intestinal calcium excretion does not rise above the small normal value during the first 3 hours. They confirmed the idea that the calcium does not leave the body at first by observing that in eviscerated and nephrectomised animals the fall of blood-calcium was just "as abrupt and profound" as in normal animals.

It thus appears probable that during the first 3 hours most of the injected calcium is deposited on the bones, but we have yet to explain the large disappearance in the first few minutes.

Precipitation as phosphate following toxic doses is envisaged by Heubner [1923, 1931]. On the other hand, while injection of phosphate causes a hypocalcaemia [Binger, 1917], large injections of calcium actually cause a rise in inorganic phosphate [Salvesen, Hastings and MacIntosh, 1924; Collip, 1926]. Brull [1930] added calcium chloride to serum circulating in a Starling

heart-lung preparation: there was no precipitation or alteration of the inorganic phosphate; some of the added calcium became non-diffusible. The writer has shown *in vivo* (Table VII) that a few minutes after the injection of calcium levulinate the inorganic phosphate is not lowered. Earlier in the paper it was shown that there was no calcium in the form of a suspension, which can be centrifuged. There seems, therefore, no evidence of the immediate precipitation of calcium phosphate.

The immediate precipitation of injected calcium chloride as carbonate according to the equation

$$CaCl_2 + 2NaHCO_3 \rightarrow 2NaCl + CaCO_3 + CO_2 + H_2O$$

would of course cause a decrease of alkali reserve. Acidosis is said to be caused by injection of calcium chloride owing to excretion of calcium through the intestine [Mainzer, 1927]. In Exp. 13 (Table VIII) about 5 minutes after a large injection of calcium chloride the alkali reserve had diminished by 14 %, corresponding to a precipitation of 3·6 mg. Ca/100 cc. serum. This figure is however too high, as a dilution of the blood of at least 7·4 % had occurred (haemoglobin comparison). Using the high figure of 9 % for the blood-volume, 28 mg. Ca/100 cc. had disappeared in the 5 minutes. There is clearly little precipitation as carbonate.

There remains the possibility of immediate rapid diffusion into the tissue spaces. Following the work of Chiari and Januschke [1910] and Meyer [1910], it was believed that calcium decreased the permeability of the capillary walls; that such is not the case to any extent has been shown by Yanagawa [1916], Bayliss [1918], and Smith and Mendel [1920]. That calcium can readily penetrate the capillary walls is shown by the appearance of calcium in oedema fluid and serous transudates; while Arnold and Mendel [1927] concluded that calcium passes easily and rapidly at all times between blood, lymph and tissues.

We are thus led to picture the following events: immediately following the injection of hypertonic calcium salt solutions into the blood-stream, calcium diffuses into the tissue spaces until there is some form of equilibrium between the calcium in these spaces and the raised blood-calcium. The added calcium now disappears from the tissues and from the blood-stream, the rate of disappearance from the latter being approximately proportional at any instant to the excess of blood-calcium above the normal value, until at the end of 2–3 hours (when the tissue analyses of Heubner and Rona, *etc.* were made) the calcium content of tissues and blood has returned to normal. The calcium appears for the most part to be deposited in the bones, but a smaller quantity is probably simultaneously excreted.

There is some doubt concerning the fate of the injected calcium after this, for varying results have been obtained. Dadlez [1926] claimed that the retention of calcium in tissues or bones immediately following the injection is only temporary, a complete excretion of the injected calcium being achieved

in 24 hours. On the other hand, Greenwald and Gross [1925] recovered in 6 days only 0·40 g. of the 0·76 g. which was injected, while Salvesen, Hastings and MacIntosh [1924] even state that the faecal excretion of calcium during the 6 days following a large injection of calcium chloride is lower than that before the injection. Presumably, the relation of the amount of calcium in the diet to the demands of the animal is a factor which has a profound influence in this matter.

SUMMARY.

1. The serum-calcium as determined by direct precipitation can give an accurate measure of the calcium content of the blood following the intravenous injection of calcium levulinate.

2. Following the intravenous injection of calcium chloride and of calcium levulinate into cabbage-fed rabbits, it was found that the rate of fall of serum-calcium at any time greater than 5 minutes after the injection was approximately proportional to the excess at that time of the serum-calcium above the final constant level. In addition, there was a large disappearance of serum-calcium during the first 5 minutes not accounted for by this relationship.

3. The bearing of the experimental results upon our knowledge of the fate of intravenously injected calcium is discussed, and a description is given of the events which probably occur.

It is a great pleasure to acknowledge the interest taken by Prof. E. C. Dodds in these experiments.

The work was done under a research grant from the Crookes Laboratories, for which the writer wishes to make acknowledgment here.

REFERENCES.

Arnold and Mendel (1927). *J. Biol. Chem.* **72**, 189.
Bayliss (1918). Intravenous injection in wound shock, p. 34. (Longmans, Green and Co., London.)
Bennett (1931). *Lancet*, ii, 403.
Binger (1917). *J. Pharm. Exp. Ther.* **10**, 105.
Brull (1930). *Arch. Internat. Physiol.* **32**, 138.
Chiari and Januschke (1910). *Wien. klin. Woch.* **23**, 427.
Clark (1920). *J. Biol. Chem.* **43**, 89.
—— and Collip (1925). *J. Biol. Chem.* **63**, 462.
Collip (1926). *Amer. J. Physiol.* **76**, 472.
Condorelli (1926). *Arch. Farm. Sper.* **41**, 122.
—— (1927). *Arch. Farm. Sper.* **43**, 88.
Conrad (1878). *Ber. deutsch. chem. Ges.* **11**, 2177.
Culhane (1927). *Biochem. J.* **21**, 1015.
—— (1930). *J. Biol. Chem.* **86**, 113.
Dadlez (1926). *Biochem. Z.* **171**, 146.
Davies, Dickens and Dodds (1926). *Biochem. J.* **20**, 695.
Derevici (1929). *Compt. Rend. Soc. Biol.* **100**, 925.
Dodds and Greville (1931). *Brit. Med. J.* ii, 190.
Erlanger (1921). *Physiol. Rev.* **1**, 177.
Fishberg (1930). *J. Biol. Chem.* **86**, 665.

Gerschmann (1930). *Compt. Rend. Soc. Biol.* **104**, 413.
Greenberg and Gunther (1930). *J. Biol. Chem.* **85**, 491.
Greenwald and Gross (1925). *J. Biol. Chem.* **66**, 201.
Grollman (1927). *J. Biol. Chem.* **72**, 565.
Guillaumin (1930). *Bull. Soc. Chim. Biol.* **12**, 1269.
Hecht (1924). *Biochem. Z.* **144**, 270.
Hendriks (1929). *Biochem. J.* **23**, 1206.
Hetényi (1924). *Z. ges. exp. Med.* **43**, 123.
—— and Nógrádi (1925). *Klin. Woch.* **4**, 1308.
Heubner (1923). *Klin. Woch.* **2**, 1603.
—— (1925). *Biochem. Z.* **156**, 171.
—— (1931). *Biochem. Z.* **234**, 221.
—— and Rona (1919, 1). *Biochem. Z.* **93**, 187.
—— —— (1919, 2). *Biochem. Z.* **93**, 353.
—— —— (1923). *Biochem. Z.* **135**, 248.
Jungmann and Samter (1924). *Biochem. Z.* **144**, 265.
Kapsinow and Underhill (1929). *J. Biol. Chem.* **82**, 377.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
Leiboff (1930). *J. Biol. Chem.* **85**, 759.
Loucks and Scott (1929–30). *Amer. J. Physiol.* **91**, 27.
Mainzer (1927). *Klin. Woch.* **6**, 1689.
Meyer (1910). *Münch. med. Woch.* 2277.
Myers (1924). Practical chemical analysis of blood. (Kimpton, London.)
Proverman and Brull (1930). *Bull. Soc. Chim. Biol.* **12**, 1151.
Rothlin (1930). *Z. ges. exp. Med.* **70**, 634.
Salvesen, Hastings and MacIntosh (1924). *J. Biol. Chem.* **60**, 327.
Smith and Mendel (1920). *Amer. J. Physiol.* **53**, 323.
Stewart and Percival (1927). *Biochem. J.* **21**, 301.
Taylor and Fine (1930). *Amer. J. Physiol.* **93**, 544.
Van Slyke (1917). *J. Biol. Chem.* **32**, 455.
—— and Sendroy (1929). *J. Biol. Chem.* **84**, 217.
de Wesselow (1924). Chemistry of the blood in clinical medicine, p. 244. (Benn, London.)
Widmark and Vahlquist (1931). *Biochem. Z.* **230**, 245.
Yanagawa (1916). *J. Pharm. Exp. Ther.* **9**, 75.

CCVIII. THE ABSORPTION OF WATER BY GELATIN.

PART VII. THE INFLUENCE OF TEMPERATURE ON SWELLING IN ACID SOLUTIONS AND THE COMBINATION OF GELATIN WITH HYDROCHLORIC, NITRIC OR SULPHURIC ACID.

By WINNIFRED BERTHA PLEASS.

From the Laboratories of the British Leather Manufacturers' Research Association.

(*Received October 12th, 1931.*)

It has long been known that the influence of aqueous solutions of inorganic substances on the physical properties of proteins is modified by the nature of the ions present. Loeb [1922] has stated that at the same p_H dibasic acids and diacidic bases induce only half as much water absorption by gelatin as monoacidic bases and monobasic acids.

The simple mathematical relationship postulated by Loeb has been denied by various workers: Ostwald, Kuhn and Böhme [1925], investigating the swelling of gelatin, found that the volume at maximum swelling was not related to the valency of the anion of the acid; Küntzel [1926], working on the contraction of tendons from the tail of the rat in acid solutions, and also the author [1929], working on the increase in weight of purified leaf gelatin, found that at the same p_H the influence of nitric acid is slightly greater than that of hydrochloric acid, which in turn is considerably greater than that of sulphuric acid at $p_H < 3{\cdot}2$. In very dilute solutions however no difference in the effects of the three acids can be distinguished. Isgarischev and Pomeranzeva [1926] have measured the swelling of powdered caseinogen in $N/20$ solutions of a large number of organic acids and suggest that the predominating factor is the nature and structure of the anion of the acid.

The author [Pleass, 1929] discussed the question of the magnitude of the swelling and also reported that the p_H for maximum swelling was determined by the anion of the acid. Recently published work [Jordan-Lloyd, 1930; Pleass, 1930] has shown that, although this p_H in solutions of hydrochloric acid is independent of the temperature between 0° and 18°, in solutions of sodium hydroxide and calcium hydroxide the p_H for maximum swelling becomes greater as the temperature is decreased. It was decided, therefore, that the influence of temperature on solutions of sulphuric acid should also be investigated.

The experimental material and method were exactly similar to that previously described [Jordan-Lloyd and Pleass, 1927; Pleass, 1930]. Pieces of purified leaf gelatin were weighed and immersed in solutions of sulphuric acid of varying concentrations at constant temperatures.

After 7 days' immersion at 0°, 4 days at 12°, or 3 days at 18° or 25°, the pieces of gelatin were removed from the solutions, lightly blotted and weighed. The final weights are expressed as a percentage of the initial weight, corrected to its weight after drying at 100°. The figures for the percentage weights of the gelatin are plotted graphically against the final p_H of the solutions.

In Fig. 1 are plotted the curves for the water absorption of gelatin from solutions of sulphuric acid of final p_H 0·1–5·0 at temperatures of 0°, 12°, 18° and 25°.

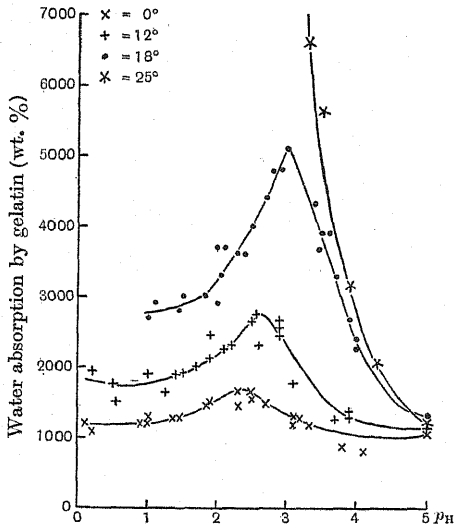


Fig. 1. The influence of temperature and p_H on the absorption of water by gelatin in solutions of sulphuric acid.

The first inspection of the curves shows that the lower the temperature, the less the water absorption. At 25°, as the p_H moves from 5·0 to 3·3, the water absorption of the gelatin increases to so great a degree that in the more concentrated solutions of sulphuric acid the gelatin is too soft to weigh. At 12° and 0°, the degree of swelling is much less than at 18°, and also at the lower temperatures the peak of maximum swelling is less sharply defined. Over the whole range of p_H studied, the magnitude of the swelling lies between 1000 and 1800 at 0°, 1100 and 2800 at 12° and 1300 and 5100 at 18°. At 0° and 12°, the gelatin does not dissolve in solutions of sulphuric acid as acid as p_H 0·1. In an analogous manner, Jordan-Lloyd [1930] found that gelatin did not dissolve in *N* hydrochloric acid at 0°, but at 18° the gelatin dissolved in all solutions of $p_H < 0·9$.

A closer examination of the curves reveals that not only is the degree of swelling and the sharpness of the peak dependent on the temperature, but

that the maximum occurs at p_H 3·0 at 18°, at p_H 2·6 at 12° and at p_H 2·4 at 0°.

The fact that the p_H for maximum swelling of gelatin in solutions of sulphuric acid varies with temperature in an analogous manner to the alteration in solutions of sodium and calcium hydroxides, while the p_H for maximum swelling in solutions of hydrochloric acid is independent of temperature (from 0 to 18°) is very striking. Jordan-Lloyd [1930] and the author [Pleass, 1930] suggested that in the alkaline systems this phenomenon might be due to some structural change in the gelatin molecule, possibly hydrolysis, which proceeded to a greater extent at the higher temperature. This opinion was based largely on the fact that gels containing sodium hydroxide decrease in p_H on keeping for several days at 18°, even when atmospheric carbon dioxide is rigidly excluded. In order to investigate more fully the possibility of the

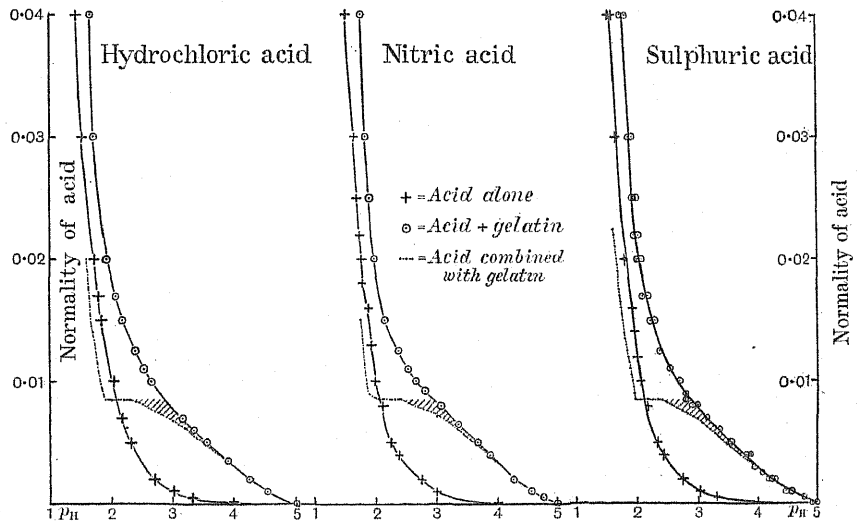


Fig. 2. Titration and acid fixation curves of a 1 % solution of gelatin with hydrochloric, nitric or sulphuric acid at 18–20°.

hydrolysis of gelatin occurring in the presence of mineral acids, the p_H of a 1 % solution of gelatin containing various known quantities of hydrochloric, nitric or sulphuric acid were determined by means of the glass electrode and plotted against the normality of the acids present (see Fig. 2) and after keeping for a week at 18–20° these gels were re-examined. It was found that within the limits of the experimental error no changes in the p_H were detected. Thus, no evidence was obtained of hydrolysis of the gelatin on keeping in any of the acid solutions.

The p_H of solutions of the acids alone were also measured and the results are plotted against the corresponding normality of the acids (Fig. 2). The amount of acid fixed by the gelatin has been derived by plotting the differences between the curve for the acids alone and the curve for acid in the presence of the gelatin at each p_H. The curves for the combination of acid are

remarkably similar for the three acids examined—in fact they are probably identical within the experimental error of the method. With all three acids, a flat portion of the curve occurs from about p_H 2·4 to 1·9 and at a normality of acid of 0·0085. This indicates that the combining weight of gelatin is the same for the three acids (approximately 1180) from p_H 2·4 to 1·9. Assuming that the gelatin behaves as a weak base and combines with strong acids in accordance with the following equation in solutions of $p_H > 1·9$

$$\text{Gelatin} + \text{HX} \rightleftharpoons \text{Gelatin HX},$$

therefore

$$\frac{[\text{G}][\text{HX}]}{[\text{G.HX}]} = \text{K}.$$

In very dilute solutions, the reaction will proceed predominantly from left to right since there is an excess of gelatin. That is, most of the acid is in combination with the gelatin (*cf.* Fig. 2 where the curve for gelatin and acid is almost superimposed on the curve for gelatin combined with acid).

In moderately dilute acid solutions, say p_H 3·5–2·5, the reaction proceeds in both directions since there is neither an excess of acid nor of gelatin to force the reaction to proceed to completion. An indication of the degree of dissociation of the gelatin-acid compound is given by the shaded area in the graphs (Fig. 2).

In more concentrated acid solutions (p_H 2·5–1·9), the excess of acid causes the reaction to proceed from left to right. The flatness of the dotted curve over this range shows that the full quota of acid for the complete neutralisation of the most strongly basic groups (probably the amino-groups) has been taken up. It can be seen that half neutralisation of the gelatin occurs at about p_H 3·7 from which it may be deduced that the first basic dissociation constant is 5×10^{-11}. This value is identical within the limits of experimental error with that obtained by Atkin and Douglas [1924] working at 24° and using the hydrogen electrode for the determinations of the p_H. In strongly acid solutions (p_H 1·9) the gelatin takes up more acid, probably by means of the acid combining with the weaker basic groups (possibly the imino-groups). This is in agreement with the work of Jordan-Lloyd and Mayes [1922] and Atkin and Douglas [1924].

Thus the p_H for maximum swelling of gelatin in solutions of hydrochloric and nitric acids at temperatures between 0° and 18° and in solutions of sulphuric acid at 0° approximates very nearly to the highest p_H at which the first stage of fixation of these acids by the gelatin at 18–20° is complete, namely p_H 2·4. Since the combination of gelatin with all three acids occurs stoichiometrically at 18–20°, the differences in the magnitude of the swelling and the p_H for maximum swelling of the gelatin in solutions of the three acids at this temperature may possibly be explained by the specific characteristics of the anions and their influence on the physico-chemical properties of water, since these differences become less as the temperature is decreased until at 0°, the freezing point of water, they are negligible.

Jordan-Lloyd has recently [1931] established that the magnitude of the water absorption of both dried and undried gelatin gels is proportional to the square of the centigrade temperature from 0 to 18° for all the external conditions studied, *i.e.* water, solutions of acids, bases and salts. She develops the idea that the influence of temperature on the activity of the water, one of the common factors in all the systems, may be the direct cause of the influence of temperature on swelling. She emphasises the point that the swelling of the gelatin is proportional to the square of the degrees on a scale of which the zero is based on a property of water.

As previously indicated [Pleass, 1930], at 0° at the p_H of maximum swelling in hydrochloric and sulphuric acids, the hydrogen ion concentration is almost equal to that of the hydroxyl ion concentration at which maximum swelling occurs at 0° in the alkaline system (p_H 11·5–11·6). On superficial examination, therefore, it would appear that, assuming that the p_H of the solutions in which maximum swelling of the gelatin occurs gives an indication of the basicity of the gelatin in acid solutions and of the acidity of the gelatin in alkaline solutions, the isoelectric point of the gelatin should coincide with the p_H of neutrality. Owing, however, to the logarithmic nature of the p_H scale, small variations in the ionic concentration at the positions of maximum swelling are inappreciable, while similar variations in ionic concentration are of considerable magnitude near the neutral point when expressed on the p_H scale.

It should also be indicated that in all the experiments on the swelling of gelatin dealt with in this series of papers, the p_H values considered have been those of the external solutions, since the determination of the p_H of the internal gel involves the melting of the gel and may be attended by unknown errors. It is probable however that an accurate knowledge of the internal p_H of the gel would be very valuable when correlating the swelling of gelatin with the combination of gelatin with acids and alkalis.

Summary.

The water absorption of purified leaf gelatin in solutions of sulphuric acid from p_H 0·1 to 5 at 0°, 12°, 18° and 25° has been studied. Increase in temperature causes an increase in water absorption until at 25° in all solutions more acid than p_H 3·2 the gelatin is too soft to weigh.

Maximum swelling occurs at p_H 3·0 at 18°, p_H 2·6 at 12° and p_H 2·4 at 0°.

Curves for the combination of gelatin with hydrochloric, nitric and sulphuric acids from p_H 5·0 to 1·7 are given. The three curves are practically identical, exhibiting a flat portion from p_H 2·4 to 1·9, and indicate a combining weight for gelatin of 1180 and a first basic dissociation constant of 5×10^{-11}.

No evidence of hydrolysis of gelatin in these acid solutions (*i.e.* $p_H > 1·5$ at 18–20°) has been obtained.

The author wishes to express her thanks to the Council of the British Leather Manufacturers' Research Association for permission to publish this work and to the Director, Dr Jordan-Lloyd, for her continued interest and advice.

REFERENCES.

Atkin and Douglas (1924). *J. Soc. Leather Trades' Chem.* **8**, 359.
Isgarischev and Pomeranzeva (1926). *Koll. Z.* **38**, 235.
Jordan-Lloyd (1930). *Biochem. J.* **24**, 1460.
—— (1931). *Biochem. J.* **25**, 1580.
—— and Mayes (1922). *Proc. Roy. Soc. Lond.* B **93**, 69.
—— and Pleass (1927). *Biochem. J.* **21**, 1352.
Küntzel (1926). *Koll. Z.* **40**, 264.
Loeb (1922). Proteins and the theory of colloidal behaviour. (New York.)
Ostwald, Kuhn and Böhme (1925). *Koll. Chem. Beihefte*, **20**, 412.
Pleass (1929). *Biochem. J.* **23**, 358.
—— (1930). *Biochem. J.* **24**, 1472.

CCIX. A METHOD FOR DETERMINING ALUMINIUM IN CERTAIN ALUMINIUM PROTEIN COMPOUNDS.

By CLIFFORD GEORGE POPE.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

(*Received October 13th, 1931.*)

In connection with the work which has been carried out at these Laboratories on the precipitation of diphtheria toxoid by various aluminium salts [Glenny, Pope, Waddington and Wallace, 1926; Wallace, 1927; Glenny and Barr, 1931] it became necessary to make a number of determinations of the aluminium content of the precipitates so formed.

From an investigation of various methods which have been proposed for the estimation of aluminium we came to the conclusion that precipitation of the aluminium by use of 8-hydroxyquinoline would prove most satisfactory for our purpose. This reagent was used by Berg [1926] for the estimation of aluminium, and later Pichler [1929] reported a micro-method based on its use.

The suspensions of alum-toxoid with which we were working contained aluminium in an amount varying from a fraction of a milligram to about 1 mg. per cc. and the method was adopted for convenience for amounts varying from 0·3 to 1·0 mg. Using less than 0·3 mg. the results were not reliable, and for choice the material tested should contain between 0·5 and 1·0 mg. aluminium.

A difficulty, regarding which we can find no mention in the literature, was encountered in dealing with the aluminium complex salt, owing to the fact that the precipitate is not easily "wetted" and behaves rather like finely divided sulphur. This property made it difficult to transfer the precipitate quantitatively to a filter. While this difficulty may have been due to the sample of 8-hydroxyquinoline used, it was found that repeated crystallisation of this reagent did not obviate the trouble. At the suggestion of Dr Trevan, we tried the effect of adding a little sodium taurocholate, which proved effective.

We found it more convenient to determine the amount of "complex" formed volumetrically instead of gravimetrically. Berg titrated the complex directly with standard bromine solution using an indicator, but in our experiments better results were obtained by dissolving the complex in hydrochloric acid, adding excess of potassium bromate, and then determining the excess bromine by adding potassium iodide and titrating with thiosulphate.

At first we used 2*N* ammonium acetate following the method of Pichler [1929] in order to precipitate the complex, but we found that, unless the precipitate was washed very thoroughly, trouble was experienced owing to traces of acetate remaining in the filter and reacting with bromine during the estimation. Borax solution was found to be much more satisfactory than acetate for our purpose, and it had the added advantage that it dissolved the alum-toxoid precipitates and so obviated the necessity for incineration with sulphuric acid. Further, as the solubility of 8-hydroxyquinoline was greater in borax solution than in water, the precipitate when collected on the Gooch crucible was washed with borax solution to remove the excess of this reagent.

Reagents.

Standard solution of aluminium. Potassium alum (A.R.) dried in a vacuum over calcium chloride. Dissolve 1·7509 g. in distilled water and make to 100 cc. (*Note.* In working with a solution made to contain 0·1 mg. aluminium per cc. it was found that after a day or two in a volumetric flask the solution gave no reaction for aluminium when tested with aurintricarboxylic acid, using the method of Myers, Mull and Morrison [1928]. After adding one drop of concentrated H_2SO_4 the aluminium, which may have been adsorbed on the glass of the flask, was liberated and correct values for the aluminium present were obtained. In view of this experience we have made it a practice to add one drop of H_2SO_4 when making up standards.) The standard solution contains 1 mg. of aluminium per cc.

Borax solution. A 5 % solution of sodium borate (A.R.).

8-Hydroxyquinoline. A 2 % solution in absolute alcohol. 1 cc. of this reagent is sufficient to precipitate 1·2 mg. of aluminium. When 1·0 cc. of this solution is mixed with 10 cc. of 5 % borax solution, a little 8-hydroxyquinoline may separate if the temperature is below 15°. This will not affect the results if the amount of aluminium is between 0·5 and 1·0 mg. but for amounts below 0·5 mg. it is better to use 0·5 cc. of reagent.

Hydrochloric acid. Hydrochloric acid (A.R.) diluted 1 in 3. For each test 40 cc. are used.

Bromide-bromate solution. Dissolve 1·6702 g. potassium bromate (A.R.) and 10 g. of potassium bromide in water and make to 1000 cc. This solution is *M*/100 in respect to the bromate, and 10 cc. provide sufficient bromine for 1·35 mg. of aluminium as $Al(C_9H_6ON)_3$.

N/20 thiosulphate solution. Prepared and standardised in the usual manner. Each cc. of *N*/20 thiosulphate = 0·1129 mg. aluminium.

Blank titration for total bromine available.

Measure out 40 cc. of HCl reagent into a beaker and add water to about 200 cc. Add 10 cc. of bromate-bromide reagent, and after two minutes 1 g. (approx.) of potassium iodide. Titrate the liberated iodine with *N*/20 thiosulphate until almost colourless, completing after the addition of starch

indicator in the usual manner. If the bromate and thiosulphate are $M/100$ and $N/20$ respectively, 10 cc. of bromate-bromide reagent will require 12 cc. of thiosulphate.

Estimation of aluminium.

Measure out an amount of the material containing between 0·3 and 1·0 mg. (The amount present may be roughly estimated from the bulk of precipitate given with the 8-hydroxyquinoline.) Add 10 cc. of borax solution, heat to boiling-point, then add 1·0 cc. of 8-hydroxyquinoline reagent. At this stage two drops of 10 % sodium taurocholate are added to cause "wetting" of the precipitate. The tubes are now heated in a boiling water-bath to drive off the alcohol in which the precipitate is somewhat soluble. They are then cooled to room temperature.

The yellow precipitate is separated by filtration through a Gooch crucible, traces of precipitate in the tube are removed, and finally the precipitate on the crucible is washed with 5 % borax solution. The precipitate is then ready to be dissolved in HCl.

Heat 40 cc. of HCl reagent to boiling. Hold the crucible over a 400 cc. beaker and half fill it with boiling HCl. Stir gently with a small glass rod, but avoid disturbing the asbestos mat at this stage. When most of the first addition of HCl has passed through the filter, add more boiling HCl and stir gently to break up the asbestos mat. Wash through with the remainder of the 40 cc. of HCl. Finally rinse the crucible with a little distilled water, wash down the sides of the beaker and make to a total volume of about 200 cc.

Add 10 cc. of bromate-bromide solution, stir well and after two minutes add about 1·0 g. potassium iodide. Titrate with $N/20$ thiosulphate. Some caution is necessary in the titration owing to the yellow colour of the bromine derivative of 8-hydroxyquinoline, and the starch indicator should, therefore, be added earlier than usual. The amount of aluminium is obtained as follows:

$$(T_1-T_2) \times 0{\cdot}1129 \times N \text{ mg. aluminium,}$$

where

T_1 = cc. thiosulphate required for bromine blank,
T_2 = cc. thiosulphate required for excess bromine (titrated as iodine),
N = Normality factor for thiosulphate and each 1·0 cc. $N/20$ thiosulphate = 0·1129 mg. aluminium.

Experimental results.

The accuracy of the method using pure solution of potassium alum.

The stock solution used for the following tests was made up to contain 1·0 mg. aluminium per cc. From this there were prepared dilutions so that each fraction of a mg. used was contained in a volume of 1·0 cc. The results given below are for estimations carried out at different times, when pure solutions were included as controls in routine estimation.

Mg. Al used	Mg. Al found	Difference in mg.
0·2	0·203	+0·003
0·2	0·200	±0·000
0·2	0·216	+0·016
0·2	0·191	−0·009
0·2	0·195	−0·005
0·3	0·305	+0·005
0·3	0·302	+0·002
0·3	0·310	+0·010
0·3	0·306	+0·006
0·3	0·310	+0·010
0·4	0·409	+0·009
0·4	0·408	+0·008
0·4	0·410	+0·010
0·4	0·412	+0·012
0·4	0·404	+0·004
0·5	0·500	±0·000
0·5	0·500	±0·000
0·5	0·501	+0·001
0·5	0·506	+0·006
0·5	0·495	−0·004
0·5	0·493	−0·007
0·5	0·491	−0·009
1·0	0·995	−0·005
1·0	0·997	−0·003
1·0	0·997	−0·003
1·0	1·002	+0·002
1·0	0·998	−0·002
1·0	0·997	−0·003

Recovery of aluminium when mixed with C. diphtheriae *culture filtrates.*

In order to determine the accuracy of the method when organic materials were present in addition to the aluminium, the following experiment was carried out.

Known amounts of aluminium were measured into tubes, and 0·5 cc. of a *C. diphtheriae* toxin filtrate added. A precipitate formed in each. 10 cc. of borax solution were then added to each tube and the aluminium estimated in the manner described. The following results were obtained, showing that the presence of organic materials did not affect the results.

Total Al present (mg.)	Al found (mg.)	Difference (mg.)
0·3	0·303	+0·003
0·3	0·298	−0·002
0·4	0·403	+0·003
0·4	0·398	−0·002
0·5	0·501	+0·001
0·5	0·507	+0·007

In a further experiment, a typical *C. diphtheriae* culture filtrate was diluted 1 in 5 with saline and mixtures made containing varying amounts of potassium alum. In each case there was formed a precipitate, small in the low alum concentration and increasing as the alum concentration increased. From these mixtures varying volumes were measured out, precautions being taken to keep the suspensions as uniform as possible during measurement. The volumes required to give a suitable amount of aluminium for estimation varied from 25 to 2 cc. of suspension. The results show the method to be suitable for the special purpose for which it was worked out.

Potassium alum in mixture %	Vol. used for estimation cc.	Mg. Al found	Mg. Al per cc. found	Mg. Al per cc. calculated
0·05	25	0·713	0·0285	0·0285
0·1	20	1·085	0·0542	0·0590
0·2	10	1·141	0·1141	0·1140
0·3	5	0·871	0·1742	0·1710
0·4	5	1·120	0·2240	0·2280
0·5	4	1·150	0·2870	0·2850
0·5	4	1·130	0·2820	
0·6	3	1·020	0·3400	0·3420
0·7	3	1·185	0·3950	0·3990
0·8	2	0·906	0·4530	0·4560

SUMMARY.

A method suitable for the estimation of amounts of aluminium between 0·3 and 1·0 mg. present in suspensions produced by adding potassium alum or other aluminium salts to *C. diphtheriae* culture filtrates has been described. The method depends on the precipitation of the aluminium with 8-hydroxyquinoline and the estimation of the amount of the latter contained in the precipitate.

I wish to express my thanks to Dr J. Trevan for his helpful interest, and to my assistant, C. Kennedy, for the care with which he has carried out a number of estimations during the course of this work.

REFERENCES.

Berg (1926). *Chem. Ztg.* **50**, 754.
Glenny and Barr (1931). *J. Path. Bact.* **34**, 131.
—— Pope, Waddington and Wallace (1926). *J. Path. Bact.* **29**, 38.
Myers, Mull and Morrison (1928). *J. Biol. Chem.* **78**, 595.
Pichler (1929). *Mikrochemie (Festschrift)*, 6.
Wallace (1927). *J. Path. Bact.* **30**, 667.

CCX. THE BODY FATS OF THE PIG.

I. INFLUENCE OF INGESTED FAT ON THE COMPONENT FATTY ACIDS.

By RAMKANTA BHATTACHARYA
and THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received October 16th, 1931.*)

An opportunity to study the component fatty acids present in the depôt fat of pigs, on similar lines to the recent examination of butter fats [Hilditch and Sleightholme, 1931] and of tallows [Banks and Hilditch, 1931], was afforded when Prof. J. C. Drummond and Captain Golding supplied us with specimens of the back fats and perinephric or leaf fats from three sets of pigs which had been fed on a control diet (i) alone or supplemented with (ii) a solid or (iii) a liquid fat.

The animals were from two litters and were fed on the experimental diet from the age of 3 months until 7 months, when they were slaughtered; the average weight at the commencement of the feeding tests was about 50 lbs., and that at death about 200 lbs.

Four pigs were fed solely on a control ration consisting of 60 % barley meal, 20 % flaked maize, 15 % soya bean meal and 5 % dried yeast (with 2 lbs. ground limestone per 100 lbs. mixture); four pigs received the control ration with 3 % of shea butter fat added; and five pigs received the control ration with 3 % of arachis oil added.

The shea butter fat was supplied through the Imperial Institute and, according to an analysis made in this laboratory, its component fatty acids were myristic (0·4 %), palmitic (8·5 %), stearic (35·9 %), oleic (49·9 %) and linoleic (5·3 %) acids. The arachis oil employed was not specifically examined, but the composition of arachis oil fatty acids is of the following order [Jamieson, Baughman and Brauns, 1921]: 6–8 % palmitic, 5–6 % stearic, 3–4 % arachidic, 3 % lignoceric, 53–61 % oleic, and 22–25 % linoleic acid. The control ration itself was estimated to contain 1·5 % of fat and 12 % of proteins.

The specimens of back fat supplied were divided in each case into two parts along the approximately central "streak" or seam of connective tissue, and the component acids of the fats in the "outer" (*i.e.* nearer the skin)

layer and the "inner" layer were determined separately: differences in composition parallel with the noticeable differences in softness of the inner and outer layers were observed in all three sets of fats. Of the perinephric fats, those from the control ration animals and from the control ration with shea fat pigs were slightly infected by moulds when received; changes due to the onset of rancidity may affect to some extent the accuracy of the linoleic acid figures in these instances.

The quantity of material available was restricted, especially as regards the back fats; but, fortunately, the mixture of fatty acids in lard is comparatively simple and we believe that the data presented below are of the usual order of accuracy for fractionation analyses. It has not, of course, been practicable, with these small quantities of mixed fatty acids, to seek for evidence of the possible presence of minor amounts of the higher saturated acids characteristic of arachis oil in the fats from pigs in the diet of which 3 % of this oil had been included. We also considered it unnecessary, for the immediate purpose, to determine the small amount (up to 0·4 %) of highly unsaturated arachidonic acid which Brown and Deck [1930] have shown to be present in normal lards.

The fats were separated from tissue, *etc.*, by extraction with hot acetone, the final traces of which were removed by heating in a vacuum at 100°. The saponification equivalent, iodine value and refractive index (at 60°) of each fat, and the setting-point of the corresponding mixed fatty acids, are collected in Table I.

Table I.

Feed	Body fat	Saponification equivalent	Iodine value	$n_D^{60°}$	Mixed acids. Setting-point
Control ration	Outer back	286·1	62·6	1·4512	38·8°
,, ,,	Inner back	286·7	55·0	1·4507	41·8°
,, ,,	Leaf (perinephric)	286·0	45·7	1·4500	44·2°
Control + 3 % shea fat	Outer back	286·1	65·2	1·4519	38·5°
,, ,,	Inner back	285·4	57·0	1·4510	41·0°
,, ,,	Leaf	284·7	43·9	1·4503	45·0°
Control + 3 % arachis oil	Outer back	283·7	68·9	1·4520	37·0°
,, ,,	Inner back	285·8	61·7	1·4508	40·1°
,, ,,	Leaf	286·7	55·1	1·4501	44·0°

The component fatty acids were estimated in each case by preliminary separation into "solid" and "liquid" acids by crystallisation of the lead salts from 95 % alcohol, followed by fractional vacuum distillation of the methyl esters of each group; the details of the procedure were the same as in the case of the corresponding analyses of beef tallows [Banks and Hilditch, 1931], except that the amount of esters handled precluded in most cases any re-fractionation of the primary ester fractions. Since the tabulation of the complete fractionation data occupies excessive space, the final results only are summarised in Table II: the figures in brackets indicate the weights of mixed fatty acids employed in each fractionation analysis.

Table II. *Component fatty acid analyses of the nine pig fats.*

Acid	Solid acids	Liquid acids	Total	Fatty acids (excluding unsaponifiable matter) % (wt)	% (mols.)
Feed: control only. Outer back fat (29·7 g.).	(38·1 %)	(61·9 %)			
Myristic	—	2·03	2·03	2·0	2·4
Palmitic	24·51	—	24·51	24·6	26·3
Stearic	10·58	—	10·58	10·6	10·2
Oleic	3·01	50·15	53·16	53·3	51·8
Linoleic	—	9·46	9·46	9·5	9·3
Unsaponifiable	—	0·26	0·26	—	—
Feed: control only. Inner back fat (88·3 g.).	(45·7 %)	(54·3 %)			
Myristic	—	1·40	1·40	1·4	1·7
Palmitic	29·51	—	29·51	29·6	31·6
Stearic	13·94	—	13·94	13·9	13·4
Oleic	2·25	44·80	47·05	47·2	45·6
Linoleic	—	7·87	7·87	7·9	7·7
Unsaponifiable	—	0·23	0·23	—	—
Feed: control only. Leaf (perinephric) fat (97·1 g.).	(51·0 %)	(49·0 %)			
Myristic	—	3·65	3·65	3·6	4·4
Palmitic	28·40	—	28·40	28·5	30·2
Stearic	21·33	—	21·33	21·4	20·5
Oleic	1·27	39·95	41·22	41·3	39·9
Linoleic	—	5·18	5·18	5·2	5·0
Unsaponifiable	—	0·22	0·22	—	—
Feed: control + 3 % shea fat. Outer back fat (48·75 g.).	(37·3 %)	(62·7 %)			
Myristic	—	3·64	3·64	3·7	4·4
Palmitic	24·93	1·05	25·98	26·1	27·8
Stearic	9·42	—	9·42	9·5	9·1
Oleic	2·95	47·15	50·10	50·3	48·6
Linoleic	—	10·37	10·37	10·4	10·1
Unsaponifiable	—	0·49	0·49	—	—
Feed: control + 3 % shea fat. Inner back fat (30·7 g.).	(41·5 %)	(58·5 %)			
Myristic	1·09	3·57	4·66	4·7	5·5
Palmitic	26·50	—	26·50	26·6	28·2
Stearic	10·01	—	10·01	10·0	9·6
Oleic	3·72	47·22	50·94	51·0	49·2
Linoleic	—	7·71	7·71	7·7	7·5
Unsaponifiable	0·18	—	0·18	—	—
Feed: control + 3 % shea fat. Leaf (perinephric) fat (73·2 g.).	(50·1 %)	(49·9 %)			
Myristic	0·07	2·75	2·82	2·8	3·3
Palmitic	30·84	—	30·84	30·8	32·7
Stearic	17·87	—	17·87	17·9	17·2
Oleic	1·32	43·57	44·89	44·9	43·3
Linoleic	—	3·58	3·58	3·6	3·5
Unsaponifiable	—	—	—	—	—
Feed: control + 3 % arachis oil. Outer back fat (50·2 g.).	(37·1 %)	(62·9 %)			
Myristic	—	1·53	1·53	1·5	1·9
Palmitic	23·47	0·69	24·16	24·3	26·0
Stearic	8·95	—	8·95	9·0	8·7
Oleic	4·68	47·20	51·88	52·2	50·7
Linoleic	—	12·93	12·93	13·0	12·7
Unsaponifiable	—	0·55	0·55	—	—

Table II (*cont.*)

	Feed: control + 3 % arachis oil. (41·7 %)	(58·3 %)	Inner back fat (74·0 g.).		
Myristic	0·16	1·37	1·53	1·5	1·8
Palmitic	24·38	—	24·38	24·5	26·2
Stearic	13·67	—	13·67	13·7	13·3
Oleic	3·49	45·55	49·04	49·3	47·9
Linoleic	—	10·98	10·98	11·0	10·8
Unsaponifiable	—	0·40	0·40	—	—
	Feed: control + 3 % arachis oil. (45·3 %)	(54·7 %)	Leaf (perinephric) fat (117·7 g.).		
Myristic	—	1·70	1·70	1·7	2·0
Palmitic	24·98	0·58	25·56	25·6	27·4
Stearic	18·16	—	18·16	18·2	17·5
Oleic	2·16	42·03	44·19	44·2	43·0
Linoleic	—	10·29	10·29	10·3	10·1
Unsaponifiable	—	0·10	0·10	—	—

Discussion.

It is desirable, as in previous communications, to consider the constitution of these various fatty acid mixtures from the point of view of molecular proportion rather than weight percentage. In Table III the molar characteristics of the mixed fatty acids are arranged primarily so as to display the variations in the corresponding depôt fats due to the different diets: section (i) shows the molar distribution of the individual acids, and in section (ii) column I gives the total molar percentage of saturated acids, column II that of the combined stearic, oleic and linoleic acids, column III that of stearic acid, and column IV the molar proportion of linoleic in the unsaturated acids present.

Table III.

(i) *Molar distribution of individual acids.*

Fat	Feed	Myristic	Palmitic	Stearic	Oleic	Linoleic
Outer back	Control	2·4	26·3	10·2	51·8	9·3
,,	,, +3 % shea	4·4	27·8	9·1	48·6	10·1
,,	,, +3 % arachis	1·9	26·0	8·7	50·7	12·7
Inner back	Control	1·7	31·6	13·4	45·6	7·7
,,	,, +3 % shea	5·5	28·2	9·6	49·2	7·5
,,	,, +3 % arachis	1·8	26·2	13·3	47·9	10·8
Leaf (perinephric)	Control	4·4	30·2	20·5	39·9	5·0*
,,	,, +3 % shea	3·3	32·7	17·2	43·3	3·5*
,,	,, +3 % arachis	2·0	27·4	17·5	43·0	10·1

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Fat	Feed	I	II	III	IV
Outer back	Control	38·9	71·3	10·2	15·1
,,	,, +3 % shea	41·3	67·8	9·1	17·1
,,	,, +3 % arachis	36·6	72·1	8·7	20·0
Inner back	Control	46·7	67·7	13·4	14·3
,,	,, +3 % shea	43·3	66·3	9·6	13·1
,,	,, +3 % arachis	41·3	72·0	13·3	18·3
Leaf (perinephric)	Control	55·1	65·4	20·5	11·5*
,,	,, +3 % shea	53·2	64·0	17·2	7·5*
,,	,, +3 % arachis	46·9	70·6	17·5	18·9

* These figures may be slightly low, owing to rancidity.

The data in Table III show certain similarities and certain differences when compared with the corresponding figures for tallows [Banks and Hilditch, 1931, Tables VIII, IX, X]. In the depôt fats of both cattle and pigs, palmitic acid is present in relatively the most constant proportions (25–33 mols. %). Furthermore, in both classes, variation in the proportion of saturated to unsaturated acids as a whole (column I) is not accompanied by anything approaching the same variation in the total content of C_{18} acids (or, therefore, of palmitic and myristic acids): whilst the extreme limits of total saturated acid content lie between 36·6 and 55·1 mols. %, those of the total C_{18} acid group range only from 64·0 to 72·1 mols. %. Thus, both in the pig body fats and in the tallows, variation in the amount of unsaturated acids is mainly compensated for by corresponding changes in the stearic acid content.

Differences between the pig and cattle depôt fats consist chiefly in the lower content of myristic acid in most of the former class and especially in the higher proportion of linoleic acid present. Thus, whilst in tallows the relative amount of linoleic acid rarely exceeds 10 %, and is frequently only about 5 %, of the combined oleic and linoleic acids, the corresponding proportions in the pig fats studied range from 10 % or a little less to as much as 20 %: also, the proportion of linoleic acid (i) clearly increases with increase of unsaturation in fatty oil forming part of the diet and (ii) also increases (at all events when unsaturated fatty oils are absent from the diet) from the perinephric fat to the outer layers of the back fat.

The general effects produced by the inclusion of either a hard or a soft fat in the diet may next be considered. The shea butter fat used contained 45 mols. of saturated acids with 55 mols. of unsaturated acids, that is, it was less saturated than the inner back or leaf fats from the control-fed pigs. Its ingestion caused little alteration in the total saturation of the depôt fats, but the total C_{18} acid content was somewhat lower than in the corresponding control fats, so that the palmitic and (especially) the myristic acid contents increased (although the relative proportion of palmitic (with myristic) to stearic acid in the shea fat itself is far below the corresponding ratio in any of the pig fats).

The arachis oil feed had a very marked influence upon the total unsaturation of the fats, which increased in each case (the alteration being greatest in the leaf fat and least in the outer back layer), and also upon the relative proportions of linoleic and oleic acid. The unsaturated acids contained about 20 % of linoleic acid, this proportion being approached in the leaf and inner back fats as well as in the outer back layer. On the other hand, increase in unsaturation in the arachis oil feed experiments was remarkably closely balanced by diminution in stearic acid—the molar percentages of palmitic (with myristic acid) being 29·4, 28·0 and 27·9 in the leaf, inner and outer back fats respectively.

It is noticeable that alteration in the diet had relatively less effect on the composition of the outer layer of back fat than on that of the inner layer or

of the leaf. The analyses, also, point to an essential difference between the average composition of the fat between the "streak" and the skin, and that of the fat on the inner side of the "streak." The former is definitely the most unsaturated and also contains the highest proportion of linoleic to oleic acid: the latter approximates more nearly in composition to its corresponding perinephric fat than to the outer back fat, although it is in general less saturated than perinephric fat. The data may perhaps be summed up in the statement that, whilst ingested fat has a marked effect on the character of the leaf and inner back fats, that of the outer layer of back fat is, to a considerable degree, similar in spite of such variation in diet as is represented by the present series of feeding experiments.

The relative constancy of the outer back fat in composition may, of course, be determined by adjustment of the fat nearest the skin to a more or less constant consistency adapted to the average temperature conditions of the external atmosphere. Henriques and Hansen [1901] give details of the melting-points and iodine values of body fats similar to our figures and conclude that these characteristics are mainly determined by the external temperature. It is desirable, in this connection, to ascertain whether the layers of fat on either side of the "streak" are homogeneous, or whether there is a progressive alteration as the outer skin is approached: it is hoped in due course to examine the whole of the back fat from a single animal, divided into two or more sections on each side of the "streak," in order to settle this point.

Some further observations must be offered with reference to the tendency to constant molar content of the total C_{18} acids (or, alternatively, of the saturated acids other than stearic), which has now been remarked in the case of the component fatty acids of butter-fats, tallows and lards. In the tallows it was pointed out that the available data showed the possible existence of two classes, one containing about 62 mols. %, and the other about 70 mols. %, of total C_{18} acids. The present series of analyses takes us a little further: the range of this figure, for the pig fats now studied, is from 64·0 to 72·1 mols. % of C_{18} acids, and, broadly speaking, fats with a high content of oleic and linoleic acids have values towards the upper limit for the total C_{18} acids, and conversely. In other words, although the general constancy of the total C_{18} acid content is marked in comparison with the variation in total unsaturated acid content, yet, within this generalisation, it seems clear that increase of oleic and linoleic acids is not completely balanced by diminution in stearic acid, but that, to a subordinate extent, the total C_{18} acid content is swollen and that of the other saturated acids, palmitic and myristic, somewhat diminished.

The results given in this paper thus support the tentative suggestions offered at the conclusion of the discussion of the glyceride structure of tallows [Banks and Hilditch, 1931], namely:

(i) that ingested vegetable fats are utilised by disposal of excess of linoleic and oleic acids by oxidation, leaving a residue of palmitic (with minor quan-

tities of myristic and stearic), oleic and linoleic acids more or less adapted to the requirements of the particular storage fat (conversion of carbohydrates, *etc.*, to fat is of course another, but separate, source of depôt fats);

(ii) that the stearic acid content of body-fats, which is relatively much greater than that of the fats present in a vegetable diet, is secured by processes involving "saturation" of unsaturated acids.

It further appears that, in the case of the pig, the amount of linoleic acid tolerated in the storage fat is considerably greater than in tallows, and, moreover, that it is in some degree related to that ingested in the diet; whilst inspection of the data in Table III shows that there is some evidence that, if stearic acid results from saturation of unsaturated acids, in the pig fats it is almost entirely oleic acid, and not linoleic acid, which diminishes as the content of stearic acid rises[1].

These views accord in general, if not in all details, with those at which Ellis and co-workers have arrived in the course of their recent studies on soft pork. The percentage of each individual component acid was determined in the cases of relatively few of the numerous samples of fat dealt with by these investigators, but we have found it of great interest to convert such data, where available, into molar proportions for comparison with our own results.

In a study of the influence of a ration low in fat upon the composition of the body fat of hogs, Ellis and Zeller [1930] quote individual acid figures for the composite body fat of hogs of varying age and weight. These are reproduced, in the form of molar proportions, in Table IV.

Table IV. *Composite body fats of hogs of varying age, fed on rations low in fat [Ellis and Zeller, 1930].*

(i) *Molar distribution of individual acids.*

Hog No.	Age (days)	Live wt. (lbs.)	Fat I. V.	Myristic	Palmitic	Stearic	Oleic	Linoleic
5	110	73	61·2	1·4	27·4	8·2	56·5	6·5
7	134	110	57·4	0·9	29·9	8·7	56·0	4·5
11	246	243	53·3	1·3	28·0	11·1	58·8	0·8
13	257	282	55·1	0·9	27·2	10·7	59·9	1·3

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Hog No.	I	II	III	IV
5	37·0	71·2	8·2	10·3
7	39·5	69·2	8·7	7·4
11	40·4	70·7	11·1	1·4
13	38·8	71·9	10·7	2·2

The fat content of the diet in this series did not exceed 0·5 %, whereas in the control feed of the pigs whose body fats we have analysed there was

[1] Ellis and Harkins [1925] express the opinion that linoleic acid is not so essential a factor in adipose tissue as is oleic acid, since the amount appears to be controlled by the amount furnished in the feed; whereas deposited linoleic acid is usually less than the total amount ingested, the amounts of total saturated acids and of oleic acid are greatly in excess of the supply in the feed.

1·5 % of fat, and the pigs were killed when about 210 days old and at an average live weight of about 200 lbs. The component fatty acids of the outer back fat of pigs fed on our control diet are almost identical with the mean of Ellis and Zeller's figures for hogs 7 and 11, and it will be noticed that the nearly constant total C_{18} acid molar content in Table IV (69·2–71·9 %) accords exactly with our observations. The chief difference is in the proportion of linoleic acid, which is lower than in our experience and finally diminishes to 2 % or less of the unsaturated acids; it may well be, therefore, as Ellis and Zeller suggest, that the linoleic acid in pig depôt fat is derived exclusively from assimilated vegetable linoleic acid.

Ellis and Isbell [1926] investigated the influence of ingested fats in a somewhat drastic manner by feeding pigs (*a*) on brewer's rice, tankage and grass, (*b*) on corn, skim milk and grass, (*c*) on soya beans alone and (*d*) on arachis nuts alone. Their figures for component fatty acids (reduced to molar proportions as in Table IV) are given in Table V.

Table V. *Body fats of hogs fed on various fatty diets [Ellis and Isbell, 1926].*

(i) *Molar distribution of fatty acids.*

Feed	Fat from	I. V.	Myristic	Palmitic	Stearic	Arachidic	Oleic	Linoleic	Linolenic
Brewer's rice, *etc.*	Back	52·6	2·1	28·2	11·7	—	56·8	1·2	—
Corn, *etc.*	Meat	58·8	0·8	27·0	12·3	—	53·0	6·9	—
Soya beans alone	Back	90·7	0·9	18·7	9·2	—	39·6	31·4	0·2
,,	Back	100·6	0·4	15·3	7·7	—	38·2	37·9	0·5
Arachis (Virginian)	Meat	84·1	0·5	16·8	7·3	0·2	55·9	19·3	—
(Spanish)	Meat	91·8	0·1	11·3	4·8	0·3	63·9	19·6	—

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Feed	Fat from	I. V.	I	II	III	IV
Brewer's rice, *etc.*	Back	52·6	42·0	69·7	11·7	2·1
Corn, *etc.*	Meat	58·8	40·1	72·2	12·3	11·5
Soya beans alone	Back	90·7	28·8	80·4	9·2	44·3
,,	Back	100·6	23·4	84·3	7·7	49·8
Arachis (Virginian)	Meat	84·1	24·8	82·5	7·3	25·7
,, (Spanish)	Meat	91·8	16·5	88·3	4·8	23·5

This remarkable series shows that, whilst a balanced ration containing not more than 5 % of vegetable fat leads to body fats of exactly the same type as those previously discussed (Tables III and IV), a ration of vegetable seeds alone leads to the following effects: (*a*) the complete breakdown of any constancy in the total C_{18} acids, and consequently considerable diminution in the molar content of palmitic acid, (*b*) increase in the linoleic acid to proportions approaching those in the ingested fats and (*c*) failure to produce stearic acid to compensate for the reduction in palmitic acid or, indeed, to the normal amount of 10–20 mols. % observed with an ordinary balanced diet. Ellis and Isbell attach some significance to the approximate ratio of

2 : 1 for palmitic and stearic acids in these extreme cases, but examination of the wider range of data in Tables III and IV proves that this ratio may range from over 3 : 1 to below 1·5 : 1; in our opinion these relative proportions are not so material as the general relationships between the total C_{18} acid content and that of palmitic (with myristic) acids, coupled with the balance, within the C_{18} group, between stearic and the unsaturated acids (especially oleic acid).

The concentration of fat in the diet beyond which the tendency to constant proportions of palmitic (with myristic) and of total C_{18} acids clearly disappears is further established by the recent experiments of Ellis, Rothwell and Pool [1931] in which pigs were fed on a basal diet of hominy, tankage, alfalfa meal and mineral mixture (containing less than 1 % fat) supplemented by varying amounts of cotton-seed oil, the component fatty acids of which are myristic (0·5 %), palmitic (22 %), stearic (2 %), oleic (30·5 %) and linoleic (45 %). Their results are reduced to molar proportions in Table VI.

Table VI. *Composite back fats of hogs fed on varying rations of cotton-seed oil [Ellis, Rothwell and Pool, 1931].*

(i) *Molar distribution of fatty acids.*

Feed	Fat I. V.	Myristic	Palmitic	Stearic	Oleic	Linoleic
Basal diet only	60·6	2·0	27·3	13·2	48·8	8·7
„ + 4 % C.S.O.	60·5	1·4	26·8	20·4	38·4	13·0
„ + 8 % „	64·4	1·0	23·5	22·6	35·0	17·9
„ +12 % „	77·4	1·3	15·0	25·9	31·3	26·5

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Feed	Fat I. V.	I	II	III	IV
Basal diet only	60·6	42·5	70·7	13·2	15·2
„ + 4 % C.S.O.	60·5	48·6	71·8	20·4	25·1
„ + 8 % „	64·4	47·1	75·5	22·6	33·8
„ +12 % „	77·4	42·2	83·7	25·9	45·8

Thus, at some point between 4 and 8 % of added fat in the diet, the total C_{18} acid content ceases to be approximately constant and rises considerably, although in this instance the molar content of the C_{18} acids in the ingested cotton-seed oil itself is only 75·8 %. (It is noteworthy that, with the larger amounts of cotton-seed oil, the stearic acid content of the pig fats continued to augment, in contrast to the observations with animals fed on soya beans or arachis nuts; the parallel differences in the diet, compared with that of these oil seeds alone, are (i) the presence throughout of a substantial, if reduced, proportion of basal meat ration and (ii) the presence in the cotton-seed oil acids of about 24 mols. % of palmitic acid.)

The definite approach to a constant balance between palmitic (with myristic) acids and the total C_{18} acids which we have established in the cases of the body fats of pigs and cattle and of the milk fat of cows fed on what

may be termed a "normal" diet, coupled with the breakdown of this balance in the pig fats examined by Ellis and collaborators when more than a certain proportion of added fat was present in the diet, may be regarded in another way: a molar content of 30 % palmitic (with myristic) acid is attained in the pig depôt fats only when the quantity of ingested fat in the diet is small (not exceeding about 5 %). This is additional, if indirect, evidence that a considerable proportion of the normal depôt fat must be derived by synthesis in the animal from the non-fatty components of the diet, rather than by direct assimilation of fat. This deduction appears to be of at least equal importance, as regards the normal animal metabolic processes, to the more obvious one that, with an excessive fatty diet deficient in carbohydrates, *etc.*, the pig is capable, *faut de mieux*, of utilising directly much of the ingested fats for storage.

Lack of material, as stated previously, prevented us from including a study of the glyceride structure of the nine fats which we have examined. A full examination of the component fatty acids and glyceride structure of the corresponding fats from a single pig fed on a controlled diet is, however, nearly complete, and the additional data which will then be available will enable us to offer a possible general explanation of some of the features connected with the elaboration of animal fats and of the fundamental differences in glyceride structure between these and the vegetable seed fats.

Summary.

The component fatty acids of the perinephric fats and the outer and inner layers of the back fats from pigs fed on a basic diet (i) alone or supplemented by 3 % of (ii) shea butter or (iii) arachis oil have been determined. The general relationships which were recently observed amongst the various classes of fatty acids of tallows are found to be present also in the pig body fats, more especially a tendency to approximate constancy in the molar content of C_{18} acids in spite of variation in the total proportion of saturated to unsaturated acids; increase in saturation is mainly produced by increase of stearic acid and corresponding diminution in oleic acid.

The composition of the outer layer of back fat differed in each case from that of the inner layer, the latter being intermediate in type between the former and the perinephric fat, which it more closely resembled. The outer layer of back fat was the most unsaturated, but the differences between the three depôt fats were much reduced when the diet of the animals included arachis oil.

As regards the influence of ingested fat in the diet, the body fats from animals whose feed included the relatively saturated shea butter were not greatly different from those of the control-fed animals, but presence of arachis oil in the diet led definitely to increase of unsaturation in the stored fat and also to increase in the proportion of linoleic acid in the unsaturated acids.

Consideration of the experimental data in conjunction with the results of Ellis and collaborators on the feeding of pigs with rations containing high proportions of vegetable fat confirms our previous conclusions as to the general rules governing the composition of depôt fat of animals fed on a balanced diet, and shows clearly the amount of added fat (beyond about 5–8 % of the diet) which leads to the breakdown of these characteristics. Further, the data as a whole prove that much of the normal depôt fat of the pig must be derived from carbohydrate or other non-fatty part of the feed.

We are most grateful to Prof. Drummond, Captain Golding and the Director of the Imperial Institute for having assisted us to obtain the material for these observations; and we would also tender our thanks to the Government of the Central Provinces of India for a scholarship held by one of us (R. B.) during the course of the work.

REFERENCES.

Banks and Hilditch (1931). *Biochem. J.* **25**, 1168.
Brown and Deck (1930). *J. Amer. Chem. Soc.* **52**, 1135.
Ellis and Harkins (1925). *J. Biol. Chem.* **66**, 101.
—— and Isbell (1926). *J. Biol. Chem.* **69**, 239.
—— Rothwell and Pool (1931). *J. Biol. Chem.* **92**, 385.
—— and Zeller (1930). *J. Biol. Chem.* **89**, 185.
Henriques and Hansen (1901). *Skand. Arch. Physiol.* **11**, 151.
Hilditch and Sleightholme (1931). *Biochem. J.* **25**, 507.
Jamieson, Baughman and Brauns (1921). *J. Amer. Chem. Soc.* **43**, 1372.

CCXI. THE LIBERATION OF ELEMENTARY NITROGEN BY BACTERIA.

By NEWTON WOOD BARRITT.

From the General Microbiology Department, Rothamsted Experimental Station.

(*Received October 19th, 1931.*)

The loss of nitrogen during the putrefaction of organic matter has been the subject of numerous investigations during the past 100 years. In a review of the literature of the subject by Jensen [1906] there appears to be a difference of opinion as to the source of the nitrogen loss and of the conditions which give rise to it. Thus Reiset [1856], Dehérain and Dupont [1898] and Gibson [1895] found that nitrogen was a definite end-product in the putrefaction of meat. Lawes, Gilbert and Pugh [1861] also found that nitrogen was evolved in the aerobic decomposition of vegetable matter, and Schloesing [1889, 1] tried to confirm this result but not very successfully.

This conclusion was not supported by the work of Hüfner [1876], Kellner and Yoshi [1888], Tacke [1887] and Immendorff [1892]. These authors found that the liberation of free nitrogen only occurred in the presence of nitrites or nitrates. Jensen is of opinion that the results of the earlier workers are not reliable owing to faulty analytical methods.

Löhnis [1926] states that a loss of nitrogen from organic matter occurs only in the presence of air, and that it has not yet been proved that free nitrogen is derived from the amino-group or ammonia. He suggests the possibility of a liberation of nitrogen by the purely chemical interaction between amines or NH_3 and nitrous acid thus:

$$R.NH_2 + HNO_2 = R.OH + N_2 + H_2O$$
$$NH_3 + HNO_2 = N_2 + 2H_2O$$

This possibility was first put forward by Dietzell [1882], though the reaction was first discovered by Piria [1849]. Fowler and Kotwal [1924] found it to be ineffective except in the presence of mineral acid. Grimbert [1899], however, suggested that a liberation of amino-nitrogen may occur in a nitrate-broth medium without the development of acidity or perhaps of only local and temporary acidity.

Russell [1927] agrees with Jensen as to the unreliability of the analytical methods used by the earlier workers but considers the results of Lawes, Gilbert and Pugh [1861] and of Pfeiffer *et al.* [1897] as sufficient evidence of the possibility of nitrogen being a final product of protein breakdown. In collaboration with Richards [1917], however, he showed that no nitrogen

was evolved in the decomposition of dung under completely aerobic or completely anaerobic conditions but only under a combination of the two conditions. He accounted for the result of Lawes, Gilbert and Pugh and others by the formation under anaerobic conditions of unstable intermediate products which evolve nitrogen on contact with air. Waksman [1927] in his review of the literature does not refer to the possibility of the liberation of free nitrogen without denitrification.

Since the work of Lawes, Gilbert and Pugh, and of Pfeiffer on the liberation of nitrogen from organic matter by oxidation, three authors have recorded the same phenomenon under anaerobic conditions. Wood and Willcox [1897] obtained considerable quantities (150 cc.) of nitrogen from a peptone-glucose medium inoculated with a pure culture of a bacillus isolated from fermenting bran used in the bating of hides. Unfortunately this experiment is not above criticism since oxygen gas was also collected, which, as a product of anaerobic fermentation, suggests the leakage of air into the apparatus. Harden [1901], using a peptone-glucose medium inoculated with *B. coli*, obtained a small amount of nitrogen (7·5 cc.) but none in absence of glucose. The rôle of the sugar in promoting the evolution of nitrogen is somewhat obscure, since carbohydrates are known to have a depressing effect on ammonification [Doryland, 1916], which would presumably be an intermediate stage in the formation of free nitrogen. Bach and Sierp [1923], using sewage sludge, obtained considerable quantities of nitrogen during anaerobic (?) fermentation in the presence of large volumes of tap-water. As pointed out by Buswell and Neave [1927] the results are subject to a very large error from the daily exchange of 500 cc. of tap-water containing sufficient dissolved air to account for all the nitrogen collected.

Thus, the evidence amply bears out Pfeiffer's remarks that "there is scarcely another field of research in agricultural chemistry in which we encounter contradictions so numerous and so fully unexplained."

As Jensen points out, the position regarding the formation of free nitrogen by bacteria is complicated, not only as regards the source of the nitrogen but also as to the conditions which give rise to it.

Amidst the conflicting evidence of previous workers there is one process of the liberation of free nitrogen by bacteria that is beyond question, *viz.* denitrification. According to Mair [1908], Beijerinck and Minkman [1910] and Lloyd and Cranston [1930], nitrates are reduced in three stages: (1) nitrite, (2) hyponitrite, (3) free nitrogen. It follows therefore that in all attempts to prove the liberation of nitrogen by the oxidation of amines or ammonia it is essential to ensure the absence of nitrates, nitrites and hyponitrites. The presence of nitrifying bacteria during putrefaction has been well established by numerous workers. These organisms are now recognised as taking part in biological oxidation in symbiosis with heterotrophic organisms [Mair, 1908; Barritt, 1931].

Ammonification studies have been carried out by numerous workers in

recent years, and the production of ammonia is now regarded as an essential part of the bacterial oxidation of organic matter when the C/N ratio of the substrate is not too high [Marschal, 1893; Doryland, 1916]. The yield of ammonia is quantitative and no recent worker has recorded a loss of free nitrogen. The only certain products of the biological oxidation of ammonia are nitrite and nitrate, and, since a crude inoculation of soil organisms will oxidise ammonia quantitatively to nitrous or nitric acid [Schloesing, 1889, 2], there is no reason to suppose the existence in soil of organisms capable of oxidising ammonia to free nitrogen.

These observations are amply borne out by the work of Russell and Richards [1917] on the loss of nitrogen during the decomposition of manure, and by the work of the Royal Commission on Sewage Disposal [1908]. In both cases loss of nitrogen was only recorded in the presence of nitrates, which represent the balance of the nitrification process in excess of denitrification as stated above.

Since denitrification appears to be an essential part of the process, the following investigations were undertaken with a view to obtaining more precise data on this process and also on the possible interaction of amines and ammonia with nitrous acid, a subject which has received little attention from previous workers. The experiments of Fowler and Kotwal [1924] on this point involved small gasometric measurements (2 cc.) and are not at all convincing.

Experimental.

Culture solutions of 0·5 % concentration of the following substances were prepared with the addition of 0·05 % potassium phosphate, 0·03 % sodium chloride and 0·1 % potassium nitrate: urea, uric acid, glycine, alanine, tyrosine, ammonium acetate, ammonium sulphate, dextrose + ammonium sulphate, dextrose, acetic acid and alcohol. The distilled water used was previously inoculated by shaking each litre with 1 g. of Broadbalk soil. The initial p_H was adjusted to 6·5. 200 cc. of each culture were incubated at 25° in a 300 cc. stoppered bottle for 6 weeks. Determinations of organic *plus* ammonia nitrogen were made by the Kjeldahl method, nitrites by the Griess-Ilosvay method and nitrate by the pyrogallol colorimetric method.

During the incubation period the reaction of the culture solutions was tested daily. The rise in p_H due to ammonification of organic nitrogen and liberation of alkali by the denitrification of the potassium nitrite was corrected by the addition of solid KH_2PO_4, and the lowering of p_H due to glycolysis was corrected to some extent by the addition of calcium carbonate. In this way an average p_H of 6·5 was maintained.

The formation of nitrite occurred in all cultures within 24 hours and persisted until all the nitrate had disappeared. The amount of nitrite produced varied with the different solutions, the greatest amounts (0·013 %) being recorded in the solutions containing dextrose and alcohol. The nitrite also per-

sisted in these solutions throughout the whole period of 6 weeks. The most vigorous reduction occurred in the solutions of uric acid and amino-acids, in which cases the amount of nitrite never exceeded 0·003 % and completely disappeared within 24 days. The slower rate of reduction in the carbohydrate solution is probably due to the lower p_H owing to the buffering action of the calcium carbonate being arrested by a covering of bacterial growth.

The formation of organic acids from dextrose does not require oxygen and the oxidation of these acids is very much reduced at $p_H < 6$ [Cook and Alcock, 1931]. For this reason the nitrite is more slowly reduced and therefore persists in the carbohydrate solutions for a much longer period than in the solutions containing amino-compounds. The results of the nitrogen estimations are given in Table I.

Table I.

		C/N ratio	Initial N g. per 100 cc.		Final N after incubation			Loss of N	
	Substance		organic N+NH_3	as N_2O_5	organic N+NH_3	N_2O_3	as N_2O_5	organic N+NH_3	as N_2O_5
1	Ammonium sulphate	—	0·053	0·014	0·0531	Trace	0·014	Nil	Nil
2	Urea	0·43	0·233	0·014	0·235	Trace	0·014	Nil	Nil
3	Uric acid	1·07	0·167	0·014	0·166	—	—	0·001	0·014
4	Glycine	1·71	0·0933	0·014	0·0928	—	—	0·0005	0·014
5	Ammonium acetate	1·71	0·0530	0·014	0·0523	—	—	0·0007	0·014
6	Alanine	2·57	0·0777	0·014	0·0776	—	—	0·0001	0·014
7	Tyrosine	7·7	0·0379	0·014	0·0372	—	—	0·0007	0·014
8	Dextrose + $(NH_4)_2SO_4$	10·0	0·0127	0·014	0·0124	—	0·0136	0·0003	0·0004
9	Dextrose only	14·5	—	0·014	0·00501	—	—	(increase 0·00501)	0·009
10	Acetic acid	14·5	—	0·014	0·00093	0·004	0·009	(increase 0·00093)	0·0007
11	Alcohol	18·0	—	0·014	0·00125	—	—	(increase 0·00125)	0·0127
12	Glycine + dextrose	3·5	0·0933	0·014	0·0916	0·0025	—	(loss 0·0017)	0·0115

It will be seen that there is no significant loss of organic nitrogen or ammonia in any of the solutions except No. 12 (glycine + dextrose), in which case a loss of 0·0017 g. nitrogen occurs. Comparing this result with that of No. 4 (glycine alone) it appears that the addition of dextrose to culture No. 4 (glycine alone) has resulted in a loss of organic nitrogen.

In the case of cultures Nos. 1 and 2, ammonium sulphate and urea, there was also no loss of nitrate nitrogen. Ammonification of the urea occurred, but no further change.

In cultures Nos. 3, 4, 5, 6 and 7 (of low C/N ratio) denitrification was complete in 21 days, and although these were incubated for a further period of 21 days no further loss of nitrogen occurred. Therefore the organisms concerned in the liberation of nitrogen during the first 3 weeks were unable to liberate nitrogen from either ammonia or organic nitrogen.

The higher C/N ratios of cultures Nos. 9, 10 and 11 appear to be associated with a smaller loss of nitrate nitrogen and a slight increase in organic nitrogen due to synthesis.

The loss of nitrogen in culture No. 12 appears at first sight to confirm Harden's observation of the liberation of nitrogen from protein in the presence of sugar. In the above case however nitrites were present, in which respect it differs from Harden's unless nitrates or nitrites were present as an impurity. Considerable amounts of lactic acid were formed in Harden's culture with glucose and it is well known that nitrites may react with amino-acids in presence of organic acid (*e.g.* acetic acid in Van Slyke's method of estimating amino-N). We may therefore expect a loss of amino-nitrogen in the presence of organic acids and nitrite. If nitrite was present in Harden's culture, free nitrogen should also have been formed in the peptone solution without glucose by the denitrification process. In this case however the amount of nitrogen liberated would be exactly half that formed by the interaction of amines with nitrous acid. Possibly 3·5 cc. of nitrogen is not sufficient for measurement with any degree of accuracy [see Russell and Richards, 1917]. In the cases where nitrite is present at the end of the fermentation the recorded loss of organic nitrogen might have occurred by means of the interaction between amines and nitrous acid on adding sulphuric acid for the Kjeldahl digestion. The possible loss of nitrogen at this stage was tested by adding excess nitrite to a 0·5 % solution of glycine before the acid digestion. The final organic nitrogen was identical with that of the materials without the glycine, indicating complete decomposition of the amino-group. In culture No. 12 therefore the recorded loss of organic nitrogen may have occurred during analysis and not during the fermentation.

In order to test the possibility of this reaction occurring during acid fermentation sterile solutions of glycine and sodium nitrite were prepared and varying quantities of lactic acid added. The amino-nitrogen was determined by Sørensen's method of formaldehyde titration. The results obtained are given in Table II.

Table II.

			Initial p_H	cc. $N/10$ NaOH required
0·5 % glycine + 0·5 % $NaNO_2$			6·5	31·0
,,	,,	+ lactic acid	6·2	30·6 (12 hrs.)
,,	,,	,,	5·7	30·5 ,,
,,	,,	,,	4·8	30·5 ,,
,,	,,	,,	4·2	28·4 ,,
,,	,,	,,	3·8	25·8 (5 mins.)
				7·8 (12 hrs.)

These results show that the rate of decomposition of the amino-acid is considerably diminished above p_H 4. It was afterwards found that the p_H of the less acid solutions increased rapidly, so that an initial p_H of 4·2 became 5·2 in 10 minutes and 6·5 in 1 hour. This rise in p_H is probably due to the volatility of the nitrous acid. At $p_H < 5·0$ a rapid evolution of gas occurred only on shaking the flask, and no gas appeared to be evolved when the solution remained quiescent. This liability to supersaturation with nitrogen gas must invalidate the results obtained by gasometric methods unless accompanied

by vigorous fermentation. With a continuous production of organic acid such as occurs in the fermentation of carbohydrates a more effective interaction between the amino-acid and nitrites could occur and result in a greater loss of free nitrogen.

Under strongly aerobic conditions organic acids are oxidised to carbonic acid with a consequent rise in p_H of the medium but, since nitrous acid is also liberated from nitrites by carbon dioxide [Moore, 1904], it seemed desirable to repeat these experiments with carbonic acid in place of lactic acid. Solutions of carbonic acid were obtained from a sparklet-siphon, using distilled water and liquid CO_2. A solution of calcium bicarbonate was also used. The results obtained are as follows:

Formaldehyde titrations of 0·5 % glycine + 0·5 % nitrite

	p_H	cc. $N/10$ NaOH required
In water only	6·8	31·0
In supersaturated solutions of CO_2 at atmospheric pressure 12 hours	5·8	23·2
In saturated solution of CO_2 under pressure	5·8	20·5
In calcium bicarbonate solution	6·0–6·8	30·0

These results show that it is possible to obtain a loss of nitrogen by the interaction of glycine and nitrite in a solution supersaturated with carbon dioxide. It is unlikely that such conditions would occur in the decomposition of organic matter. In the culture solutions described above (Table I) the reaction was maintained at p_H 6·5 and no loss of amino-nitrogen occurred [*cf.* Grimbert, 1899].

From these results it must be concluded that a loss of organic nitrogen is possible during denitrification provided that organic acids and amino-compounds are simultaneously produced. This event is more likely to occur when readily available carbohydrates like dextrose are present. It would also be expected to occur in the absence of nitrates when anaerobic conditions are followed by aeration. In the presence of nitrifying organisms amino-acids may be regarded as the unstable compounds referred to by Russell and Richards [1917] as oxidising to elementary nitrogen on exposure to air.

The simultaneous production of ammonia by the oxidation of protein and of nitrite by the reduction of nitrate suggests the possibility of free nitrogen being formed by the spontaneous decomposition of ammonium nitrite. This has been suggested by both Löhnis and Waksman. It is well known that ammonium nitrite is readily decomposed on heating, but in dilute solution it appears to be quite stable. A 0·5 % solution accurately prepared by mixing molecular equivalents of ammonium sulphate and sodium nitrite was incubated at 25° for 30 days without loss of either ammonia or nitrite and without a trace of gas being produced. The same result was obtained when the solution was incubated with soil. It must therefore be concluded that the decomposition of ammonium nitrite is not a source of the liberation of free nitrogen by bacteria.

SUMMARY.

1. A review of the literature shows that much confusion exists regarding the liberation of nitrogen by bacteria. The evidence of some workers that it occurs under anaerobic conditions is cancelled by that of others who show that it occurs only under aerobic conditions.

2. It is pointed out that inaccuracies in analytical methods and insufficient attention to the occurrence of nitrates or nitrification is responsible for the confusion as to the nature of the process.

3. Experiments are described which confirm the results of several previous workers that

(*a*) free nitrogen is liberated by soil bacteria during denitrification;

(*b*) there is no evolution of nitrogen from organic compounds under anaerobic conditions in the absence of oxidised nitrogen, nor from oxidised nitrogen in the absence of oxidisable organic compounds.

4. The reduction of nitrates and nitrites is effected most rapidly in the presence of compounds of low C/N ratio, and the formation of free nitrogen is strictly confined to the reduction of oxidised nitrogen provided the reaction of the medium does not fall below p_H 6·5.

5. The production of free nitrogen from organic compounds does not occur by bacterial action but only indirectly by the interaction between free nitrous acid and amino-compounds, both of which may be produced simultaneously by bacterial agency.

6. Free nitrous acid is liberated from nitrites by organic acids and carbonic acid, but does not effect the decomposition of amino-acids unless the p_H of the medium is $< 6{\cdot}0$.

7. The presence in a culture of readily available carbohydrates may result in a loss of free nitrogen from organic matter by the indirect process referred to in 5.

8. In Kjeldahl determinations of solutions of organic nitrogen the presence of nitrite results in a loss of amino-nitrogen during the acid digestion.

9. There is no liberation of free nitrogen by the decomposition of ammonium nitrite in culture solutions at ordinary temperatures.

The writer is much indebted to Mr R. G. Warren of the Chemical Department, for much useful advice and for the control of the Kjeldahl determinations, and also to Dr L. de Telegdy Kovato of Budapest (late of this department) for help in the discussion of the literature.

REFERENCES.

Bach and Sierp (1923). *Centr. Bakt. Par.* II, **58**, 401.
Barritt (1931). *Biochem. J.* **25**, 1419.
Beijerinck and Minkman (1910). *Centr. Bakt. Par.* II, **25**, 30.
Buswell and Neave (1927). *Soil Sci.* **24**, 285.
Cook and Alcock (1931). *Biochem. J.* **25**, 523.
Dehérain and Dupont (1898). *Ann. Agron.* **25**, 401.
Dietzell (1882). *Biederm. Centr. Agrik. Chem.* **11**, 417.
Doryland (1916). *N. Dakota Agric. Exp. Sta. Bull.* 116.
Fowler and Kotwal (1924). *J. Indian Inst. Sci.* **7**, 29.
Gibson (1895). *Wollnys Forschungen*, **18**, 106.
Grimbert (1899). *Ann. Inst. Pasteur*, **13**, 67.
Harden (1901). *J. Chem. Soc.* **79**, 612.
Hüfner (1876). *J. prakt. Chem.* **13**, 292.
Immendorf (1892). *Landw. Jahrb.* **21**, 281.
Jensen (1906). Lafar's Handbuch der Tech. Mykologie, III, 190. (Geb. Borntraeger, Berlin.)
Kellner and Yoshi (1888). *Z. physiol. Chem.* **12**, 95.
Lawes, Gilbert and Pugh (1861). *Phil. Trans. Roy. Soc. Lond.* 497.
Lloyd (1931). *J. Roy. Tech. Coll. Glasgow*, 530.
—— and Cranston (1930). *Biochem. J.* **24**, 529.
Löhnis (1926). Vorles. über Landw. Bakt. (Gustav Fischer, Berlin.)
Mair (1908). *J. Hygiene*, **7**, 609.
Marschal (1893). *Bull. Acad. Roy. Belgique* (3), **25**, 727.
Moore (1904). *J. Amer. Chem. Soc.* **26**, 959.
Pfeiffer, Franke, Gotze and Thurmann (1897). *Landw. Versuchs. Stat.* **48**, 189.
Piria (1849). See Cain's Chemistry and technology of the diazo-compounds, p. 1. (London.)
Reiset (1856). *Compt. Rend. Acad. Sci.* Vol. **42**.
Royal Commission on Sewage Disposal, 5th Report (1908), App. 6.
Russell (1927). Soil conditions and plant growth, p. 263. (London.)
—— and Richards (1917). *J. Agric. Sci.* **8**, 495.
Schloesing (1889, 1). *Compt. Rend. Acad. Sci.* **108**, 205.
—— (1889, 2). *Compt. Rend. Acad. Sci.* **109**, 423.
Tacke (1887). *Landw. Jahrb.* **16**, 917.
Waksman (1927). Principles of soil microbiology. (Ballière Tindall & Cox, London.)
Wood and Willcox (1897). *J. Soc. Chem. Ind.* **16**, 510.

CCXII. THE CHEMISTRY OF COOKING.

I. THE CHEMICAL CHANGES OF CARBOHYDRATES IN THE SWEET POTATO ACCORDING TO VARIOUS METHODS OF COOKING[1].

By OSAMU SINODA, SUZU KODERA and CHIYO OYA.

From the Laboratory of Household Chemistry, St Agnes' School for Girls, Kyoto.

(*Received October 21st, 1931.*)

Chemical analyses of the raw materials of our common food substances have already been made by various authors, but until now the changes of chemical composition during cooking have been little understood in spite of their evident importance from the point of view of nutritive value. The present paper represents a study of the chemical changes in the carbohydrates of the sweet potato brought about by various methods of cooking.

Method of cooking.

As the fundamental types of cooking, the authors took the following three procedures, boiling, steaming and baking. The conditions of the experiment are tabulated below. Materials were chopped and cooked just as they are prepared for the table, only without salt or any spices.

Table I. *Conditions of experiment.*

Exp. No.	A	B	C	D	E	F	G
Method of cooking	Boiling	Boiling	Boiling	Steaming	Steaming	Baking	Baking
Origin of the sample	Takatsuki Osaka-fu	Shinden Kyoto-fu	Do.	Yagi Hyogo-ken	Koma Kyoto-fu	Yagi Hyogo-ken	Koma Kyoto-fu
Weight (g.)	134	255	317	217	174	187	549
Water (cc.)	200	500	400	300	—	—	—
Added material	—	—	—	—	—	Butter 0·5	—
Time of cooking (mins.)	15	24	17	35	33	13	17
Highest temp.	100°	100°	100°	100°	100°	190°	175°

Steaming. In Exps. E and G, Kafuku's apparatus was used to measure moisture.

Baking. In Exp. F, the material (8 mm. thick) was kept at 190° for 3 minutes, and it appeared to be overbaked. In Exp. G, it was kept at 175° for 1 minute with good results.

[1] Contribution from Department of Domestic Economics, St Agnes' School for Girls (Heian Jogakuin), Kyoto, No. 2.

Gain and loss during cooking.

The sweet potato generally lost weight by cooking, especially in the case of baking, where the loss was due entirely to the removal of water.

The soluble sugar was lost in boiling or steaming, the loss in boiling being 0·4 % of the fresh material, or, about 5 % of the total soluble sugar.

Table II. *Loss by cooking.*

	Exp. No.	A	B	C	D	E	F	G
	Fresh wt.	105	98	97	98	97	63	76
	Dry wt.	—	90	95	100	99	101	101
Loss of sugars	Monosaccharides	0·6	—	—	0·2	—	—	—
	Disaccharides	0·6	—	—	0·2	—	—	—

The figures represent percentages of the respective fresh or dry weights before cooking.

Chemical changes during cooking.

It is known that starch, by the action of heat and water, is partly hydrolysed into soluble sugars. Such a partial hydrolysis of starch must be expected to occur in cooking sweet potatoes even if no water is added, as the tissue itself contains about 60 % moisture. Thus even in baking, one might expect increase in the amount of soluble sugars. Contrary to the expectation, the soluble sugars diminished considerably, and the starch-like substance increased instead. This fact is of great importance in discussing the nutritive value of the cooked food.

Table III. *Change of chemical composition by cooking.*

				Dry matter as 100				
Method of cooking	Exp. No.	Sample	Moisture %	Monosaccharides	Disaccharides	Dextrin	Starch	Cellulose
Boiling	A	Fresh	64	11·9	19·4	—	42·5	1·9
		Cooked	(67)	17·6	13·6	—	54·5	2·1
	B	Fresh	65	13·1	27·4	10·0	28·7	2·9
		Cooked	68	12·8	20·9	3·4	41·9	—
	C	Fresh	60	13·5	25·5	15·0	—	3·3
		Cooked	61	11·8	18·7	3·3	—	2·1
Steaming	D	Fresh	69	15·8	15·5	—	33·9	1·3
		Cooked	68	19·7	11·9	—	44·7	19·0
	E	Fresh	58	13·6	15·2	17·4	22·9	—
		Cooked	57	10·5	15·8	13·3	35·6	—
Baking	F	Fresh	67	12·7	20·0	—	11·9	15·0
		Cooked	47	12·5	18·1	—	11·7	1·0
	G	Fresh	62	13·4	17·1	24·5	28·9	—
		Cooked	49	12·2	14·1	17·6	38·8	—

Moisture. About 5 to 10 g. of the material were dried to a constant weight at 105°. The decrease in weight was assumed to be moisture.

Monosaccharides. About 20 g. of material was treated for 45 minutes with hot water. In the extract, reducing sugar was determined by Bertrand's method. Only in Exp. G, Willstätter's iodimetric method was adopted also.

Disaccharides. One part of the extract was hydrolysed with 3 % H_2SO_4 for 30 minutes on a water-bath. The increase in reducing power was assumed to represent disaccharides.

Dextrins. The rest of the extract was boiled with 3 % HCl for 2 hours

under reflux over a direct flame. The increase in reducing sugar less that derived from disaccharides was assumed to originate from dextrins.

Starch. The water-insoluble residue was hydrolysed by boiling with 3 % HCl for 2 hours. The reducing sugar thus formed was assumed to represent starch.

Cellulose. The residue from the starch was boiled for 30 minutes with 1 % KOH, filtered, and the solid, taken to be cellulose, was washed well with water, alcohol, and ether and dried to constant weight at 105°.

Cooking and digestibility.

The increase of insoluble polysaccharide and decrease of soluble sugars by cooking naturally suggest that cooked sweet potato would be more resistant towards the digestive enzymes. Contrary to expectation, the cooked sweet potato was more easily hydrolysable by diastase than the raw material. Further, by diastatic hydrolysis of the boiled material less dextrin and much more soluble sugar was formed.

Table IV. *Digestibility.*

A.

	Control*		Fresh material		Boiled material	
Time of digestion (hrs.)	0	2	0	2	0	2
Monosaccharides	21·7	20·3	20·5	37·5	34·3	98·8
Dextrins	49·9	55·3	51·8	78·5	56·9	68·6

B.

	Control*		Fresh material		Steamed material	
Time of digestion (hrs.)	0	2	0	2	0	2
Monosaccharides	21·9	32·1	22·6	35·6	33·3	84·2
Disaccharides	24·6	32·0	12·5	31·2	26·5	51·9
Dextrins	79·6	82·4	80·0	82·4	71·2	84·4

* Control for autolysis. Fresh material and boiled diastase solution were used.

Digestibility. The material was the same as was used in Exp. C, and that of the steamed one was the same as Exp. E. 400 cc. 1 % diastase (Pharmac. Jap.) was mixed with 20 g. of finely chopped material; in an aliquot of 150 cc. reducing sugar, disaccharides and dextrins were determined as described above. The remainder was kept for 2 hours in a thermostat at 40°, and the determination was repeated. The same was done with the steamed material. A control sample with boiled enzyme solution and fresh material was similarly treated. In measuring digestibility of steamed material, Willstätter's method was used to determine reducing sugars.

Discussion.

The peculiar increase in the starch fraction after cooking cannot be explained by loss of soluble sugars by diffusion because, even in Exp. B where the loss of dry weight is about 10 %, assuming that this 10 % loss is caused

by soluble sugars only, the apparent increase in starch cannot exceed 4 %, whilst the actual increase is, as is shown in Table III, more than 13 %.

Link and Tottingham [1923, 1925] observed a marked amount of caramelisation of sugars in plant tissues when they were heated to 80°. Since during cooking the temperature may rise as high as 230° in frying, considerable polymerisation of soluble sugars should take place, especially in the case of baking.

On the other hand, the easier digestibility means that the starch has undergone partial hydrolysis. The partially hydrolysed starch may remain in the starch fraction, if the degree of hydrolysis is slight. Thus, the apparent increase in the starch fraction and the easier digestibility after cooking can be explained by the polymerisation of soluble sugars and the partial hydrolysis of starch itself.

Daikuhara [1902] has reported that the taste of sweet potato is independent of the soluble sugar content of the raw material. It seems probable therefore that the increased sweetness of cooked sweet potato, important from a practical dietetic point of view, is connected with partial hydrolysis of the starch during cooking. Further experiments on this point are necessary.

Summary.

1. Chemical changes in the carbohydrates of sweet potato after boiling, steaming and baking were compared.

2. Sweet potato loses weight during cooking, especially by baking, owing to loss of moisture.

3. The decrease in soluble sugars and dextrins by cooking is most remarkable in baking.

4. The starch fraction always increases in cooking.

5. The digestibility of boiled and steamed material is much greater than that of the raw substance.

6. By the action of heat and water, the starch of sweet potato undergoes partial hydrolysis, whilst soluble sugars and dextrins polymerise to some extent.

The authors wish to express their sincere gratitude to Prof. Dr S. Komatsu of Kyoto Imperial University for his kind advice and valuable criticisms; to our colleagues Mrs S. Ueda and Miss U. Okamura, and pupils K. Inazumi, K. Yuhasi, K. Osikamo and N. Oka for their assistance during the experiment. Hearty thanks are due also to the Gunze Silk Filatory, Ayabe, for defraying the larger part of the cost of the experiment and to Miss H. Imamura, Sayama-mura near Kyoto, for supplying a part of the materials.

REFERENCES.

Link and Tottingham (1923). *J. Amer. Chem. Soc.* **45**, 439.
—— (1925). *J. Amer. Chem. Soc.* **47**, 470.
Daikuhara (1902). *Imper. Agric. Exp. Sta. Reports*, Tokyo, **22**, 139.

CCXIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXII. THE ISOLATION AND CHARACTERISATION OF ERGOSTEROL FROM *PENICILLIUM PUBERULUM* BAINIER GROWN ON A SYNTHETIC MEDIUM WITH GLUCOSE AS SOLE SOURCE OF CARBON.

By JOHN HOWARD BIRKINSHAW, ROBERT KENNETH CALLOW and CATHERINE FÉLICITÉE FISCHMANN.

From the London School of Hygiene and Tropical Medicine, and the National Institute for Medical Research, London, N.W. 3.

(*Received October 24th, 1931.*)

Whilst sterols are generally known to occur in moulds, and the presence of ergosterol in a number of species grown on synthetic media has been demonstrated by the development of antirachitic activity on irradiation [Preuss *et al.*, 1931], ergosterol has not previously been actually characterised after isolation from a mould grown on a synthetic medium in which glucose is the only source of carbon.

In the case of the sterol isolated from *Penicillium puberulum* Bainier, it has been found that the physical properties of the sterol itself and the benzoate prepared from it correspond closely with those of purified ergosterol from yeast and ergosteryl benzoate, respectively, and final confirmation of its identity with ergosterol is afforded by measurements of the absorption spectrum and of the antirachitic activity acquired after irradiation. These observations are significant in yielding experimental evidence of the synthesis of ergosterol from glucose.

Experimental.

The organism employed was the strain of *P. puberulum* Bainier, used by Alsberg and Black [1913] in the preparation of penicillic acid, and was supplied by the courtesy of Dr Charles Thom, of the United States Department of Agriculture. Its history is given in Dr Thom's "Penicillia," p. 272.

The medium used was a Czapek-Dox solution containing glucose as sole source of carbon, and was identical in composition with the basal metabolism solution of Raistrick and Rintoul [1931]:

Glucose	50 g.
$NaNO_3$	2 g.
KH_2PO_4	1 g.
KCl	0·5 g.
$MgSO_4,7H_2O$	0·5 g.
$FeSO_4,7H_2O$	0·01 g.
Water	1000 cc.

The cultures were grown in 1000 cc. flasks, each containing 350 cc. of medium, and were incubated at 24°, or in trays each containing 5 litres in the incubator-steriliser designed for mould cultures by Birkinshaw, Charles, Lilly and Raistrick [1931]. In the latter case the temperature was maintained at 16–20°.

In order to obtain spores for sowing, Roux bottles containing Czapek-Dox agar were inoculated from the stock culture. When a good growth of spores was attained, 150 cc. of sterile water were added to each bottle, and the surface of the culture lightly rubbed with a sterile wire. The suspension was poured into a flask, and 2·5 cc. of this suspension were used for sowing each flask, or 100 cc. for each 5 litre tray. After an incubation period averaging 2–3 weeks, corresponding with the disappearance of about 90 % of the glucose present, the mycelium was separated from the metabolism solution by filtration, and after washing with water was squeezed out, and dried by exposing in a thin layer to the air at a temperature of about 25°. When most of the water had been removed in this manner, the mycelium was further dried *in vacuo* over $CaCl_2$ or H_2SO_4. When quite brittle it was finely ground in a mortar and extracted with ether in an apparatus of the Soxhlet type. A crystalline substance could be isolated from the extract showing an intense reaction for sterols. Further work was therefore undertaken with the object of identifying the sterol or sterols present.

In a preliminary examination made of the ether-soluble material from one batch of mould the residue left after evaporation of the ether was twice recrystallised from alcohol. The product (0·35 g.) was now not completely soluble in ether. The crystals obtained from the ethereal extract (0·28 g.) had M.P. 123–133°, $[\alpha]^{20}_{5461} - 122°$ in chloroform, gave an intense blue coloration with trichloroacetic acid characteristic of ergosterol [Rosenheim, 1929] and contained 73 % of sterol (calculated as ergosterol) precipitated by digitonin.

The ether-soluble material from another batch of mould was dissolved in a large volume of light petroleum, from which three fractions were obtained. (I) on cooling, white crystals (0·55 g.), (II) after evaporation (0·70 g.), and (III) a pasty residue after complete evaporation (2·25 g.). Fraction I did not redissolve completely in ether. The residue (I *a*) (0·1 g.) had M.P. 106–121° and contained nitrogen. The ethereal extract (I *b*) was evaporated to dryness. The following data were obtained for these fractions.

Fraction	M.P.	$[\alpha]^{20}_{5461}$ in $CHCl_3$	Free sterol precipitated by digitonin %	Reaction with CCl_3CO_2H
I *b*	127–139°	−139·5°	70	Strongly +
II	65–110°	− 19·4°	7·2	Strongly +
III	Below 75°	− 6·3°	2·9	Weakly +

Fraction I *b* in 0·0025 % alcoholic solution gave an absorption spectrum similar to, but less intense than, that of ergosterol, with the following extinction coefficients: 9·9 at 290 *mμ*, 18·7 at 282 *mμ*, 18·7 at 271 *mμ*, 8·5 at 247 *mμ*. The corresponding figures for ergosterol are $\epsilon = 13·5, 25·6, 24·5, 6·6$.

These data appear to indicate that the first fraction contains the bulk of the free ergosterol, whilst fraction II probably contains ergosterol largely as ester, and fraction III consists mostly of other compounds, possibly including other sterols.

0·25 g. of fraction I *b* was boiled with 10 cc. of 3 % alcoholic potassium hydroxide for a few minutes. After the addition of a little water crystals separated on cooling. These were collected and dried over calcium chloride; weight 0·134 g., M.P. 154–158°, $[\alpha]^{20}_{5461}$ − 162° in chloroform. Pure hydrated ergosterol has M.P. 160–163°, $[\alpha]^{20}_{5461}$ − 167·2° [Callow, 1931].

The benzoate was prepared by the addition of a slight excess of benzoyl chloride to 0·11 g. of the material dissolved in 3 cc. of pyridine. The product was isolated by pouring the reaction-mixture into water and recrystallised from ethyl acetate. It separated, in the manner characteristic of ergosteryl benzoate, in leaflets changing to bulky thin needles which had M.P. 161–163°, $[\alpha]^{20}_{5461}$ − 85·3° in chloroform. Pure ergosteryl benzoate has M.P. 169–171·5°, $[\alpha]^{20}_{5461}$ − 88·3°.

Finally, the benzoate was hydrolysed, the absorption spectrum of the resultant sterol was measured, and the antirachitic activity which it acquired on irradiation was determined. In 0·002 % alcoholic solution the extinction coefficients were 15·0 at 290 *mμ*, 28·4 at 282 *mμ*, 26·9 at 271 *mμ*, and 6·2 at 247 *mμ*. The curve showed the maxima characteristic of ergosterol, but with slightly greater intensity. A solution of the sterol in alcohol (0·01 %) stirred with a stream of nitrogen was irradiated in a silica cell by a mercury vapour lamp. A solution of pure ergosterol from yeast was irradiated under the same conditions for the same period and, after dilution in olive oil, the antirachitic potencies of the two products were compared on rats by the radiographic method described by Bourdillon *et al.* [1931]. Three pairs of rats were used, to which graduated doses were given, and three rats of the same litter were used as controls and given doses of a standard solution of vitamin D. The antirachitic potencies of the two products were found to be equal.

An estimate of the yield of ergosterol from the mycelium was made in a typical experiment, using 100 flasks (*i.e.* 35 litres of medium) and incubating at 24° for 20 days. The dry mycelium weighed 391 g. and yielded an ether extract weighing 19·9 g. after removal of solvent *in vacuo*. The solid portion of the extract was collected and dried on a porous plate. It weighed 1·8 g., and in alcoholic solution gave the absorption spectrum characteristic of ergosterol. The intensity of absorption indicated the presence of 28 % of ergosterol, corresponding to 0·13 % in the dried mycelium.

Our thanks are due to Mr J. St L. Philpot for measuring the absorption spectra, which were obtained by the method described by Bourdillon *et al.* [1930].

We are also grateful to Prof. H. Raistrick and Dr O. Rosenheim for their interest in this work.

REFERENCES.

Alsberg and Black (1913). *U.S. Dept. Agric. Bur. Plant Industry Bull.* **270**, 1.
Birkinshaw, Charles, Lilly and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 127.
Bourdillon, Bruce, Fischmann and Webster (1931). *Medical Research Council Special Report No.* **158**.
—— Gaddum and Jenkins (1930). *Proc. Roy. Soc. Lond.* B **106**, 388.
Callow (1931). *Biochem. J.* **25**, 79.
Preuss, Peterson, Steenbock and Fred (1931). *J. Biol. Chem.* **90**, 369.
Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 2.
Rosenheim (1929). *Biochem. J.* **23**, 47.

CCXIV. DETERMINATION OF SMALL AMOUNTS OF PHOSPHATIDES AND CHOLESTEROL IN TISSUES[1].

BY MAURICE JOWETT AND ERIC WILLIAM LAWSON.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received October 24th, 1931.*)

ALTHOUGH there have been many publications on methods of estimating phosphatides and cholesterol in tissue, the value of many of the methods described remains uncertain. In some cases extraction of lipoid substances may be incomplete. Not all of the phosphorus in extracts may be derived from phosphatides. Colorimetric methods of doubtful validity [Nekludov and Chalatov, 1929] are still in use for determining cholesterol. While micro-gravimetric methods have been worked out for pure cholesterol, they have received little application to tissue-extracts.

Before undertaking analyses of tissues we have thought it advisable to make an investigation of methods of analysis. This is reported here with necessary details, together with such results of the analyses as are of methodical interest.

An account of analytical methods is necessary to make it possible to assess the value of the analytical data given in an accompanying paper [Jowett, 1931]. Some results of interest in themselves have also been obtained.

Extraction of lipoid substances from tissues.

In work carried out some years ago in which one of the authors took part, dried tissues were extracted with chloroform[2]. While such a solvent has the advantage that it is likely to extract little phosphorus beyond that contained in phosphatides, an impression prevails that chloroform and ether may not give complete extraction of lipoid substances.

We have therefore tested this point. Fresh rabbit liver was minced, rubbed in acetone, and dried as described later. After exhaustive extraction

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell).

[2] This method was adopted from Bullock and Cramer [1913–14], who in turn appear to have followed Smith and Mair [1911, 1913]. Dr Cramer kindly sent us a description of the methods used. The results obtained by us have been summarised in several places, *e.g.* Bell [1925, 1930] and Lewis [1927]. They are not further detailed in the accompanying paper [Jowett, 1931], since they are less accurate than the data given there.

in a Soxhlet apparatus with chloroform, the tissue was extracted with absolute alcohol[1]. The alcohol extract was taken to dryness and re-extracted with chloroform. If alcohol is assumed to complete the extraction of lipoid substances, the results show that chloroform extracts only about 82 % of the lipoids from rabbit liver.

With other tissues the proportion of the lipoids extracted by chloroform may be as low as 50 %. This is suggested by a comparison of two series of estimations on human uterine muscle, which has a fairly constant composition. In the first series, made some years ago with chloroform, the average total lipoid content found for five specimens was 3·5 %. In the present work with absolute alcohol as solvent, five specimens gave an average value of 7·2 % for total lipoids.

In the present work we have used absolute alcohol to extract lipoid substances from tissues and as the result of some preliminary experiments decided to extract the tissues without drying them thoroughly.

The tissue for analysis, weighing from 5 to 20 g., is cut up, and gross fat, connective tissue and so forth removed as far as possible. It is then finely minced in a Latapie type mincer of small size. The minced tissue is weighed after removing a portion for dry weight determination, and then covered with 20 to 80 cc. of acetone or absolute alcohol, the tissue being well stirred up immediately to avoid caking. When convenient, the material is poured into a Soxhlet thimble of double thickness, and washed with alcohol. The filtrate is retained. The thimble is mounted with wire in a conical flask under a reflux condenser, and its contents are extracted three times, for an hour each time, with boiling absolute alcohol. Fresh alcohol is used for each extraction.

The combined extracts are taken to dryness, alcohol being first distilled off, and the operation then being finished at as low a temperature as possible in a stream of carbon dioxide or nitrogen. If the temperature is too high the lipoid material obtained will not dissolve completely in chloroform; if removal of alcohol is incomplete, the chloroform extract may be turbid. The dried alcohol extract is extracted by boiling with three successive portions of chloroform for a few minutes, and the filtered chloroform extract kept for analysis.

The alcohol extraction is fairly complete. In two cases the tissue after alcohol extraction was dried, ground up and extracted with ether for 3 hours. The weight of the ether extract amounted to an additional 0·5 % for some malignant tissue, and 2·4 % for a sample of muscle.

Estimation of dry weight and total lipoid content of tissues.

Estimation of dry weight. The minced tissue is stirred with a glass rod to a uniform paste, and a portion of about 0·5–1·0 g. removed to a small, light

[1] All solvents used in this work were of good quality; before use they were dried with calcium chloride (quicklime in the case of alcohol), and redistilled using a twelve-pear column.

weighing-bottle. After being weighed, the tissue is covered with acetone, and the acetone is afterwards roughly evaporated off in a stream of nitrogen or carbon dioxide. The bottle is then dried to constant weight in the vacuum oven over sulphuric acid at 40° ; this requires about 5 days. Bottles were weighed on a micro-balance to 0·01 mg., and the change in weight of a bottle to which pure acetone was added was applied as a correction. The weights of empty bottles were reproducible within 0·2 mg., and the weights as corrected are probably more accurate than this. This would suggest that dry weight estimations are accurate within about 0·2 %, but difficulties of sampling and of reproducing the degree of dryness may make the accuracy less than that figure.

Estimation of total lipoids. The chloroform extract from a specimen of tissue is brought to a volume of 100 cc. immediately before an estimation. This is necessary, as chloroform is very expansible and volatile.

A measured volume of the extract is transferred by pipette to a weighing-bottle, which is then immersed in a water-bath at 50–55°, and a stream of carbon dioxide led over the surface of the liquid until at least 10 minutes have elapsed after evaporation of chloroform appears to be complete. After being wiped, the bottle is transferred to a desiccator containing calcium chloride and weighed soon afterwards. Every time an estimation was made, a bottle was put through the same process with pure chloroform, and its change of weight was applied as a correction to the other changes; no regular change of weight took place, and the change was usually less than 0·3 mg. The weight of lipoids taken for an estimation was from 20 to 100 mg. Duplicate estimations, which were always made, checked very closely.

Fresh estimations of the total lipoid content of the chloroform solutions were always made when further volumes of the solutions were taken for analysis[1].

The estimation of phosphatides.

It is uncertain that phosphatides are the only phosphorus-containing substances extracted from tissues by absolute alcohol, and we are relying on phosphorus estimations to determine phosphatides. We therefore undertook experiments with the object of separating phosphatides on the small scale from other phosphorus-containing substances.

According to Nerking [1910] acetone does not precipitate lecithin completely. Nor, according to experiments made by us, does cadmium chloride in alcoholic solution completely precipitate small amounts of lecithin.

Nerking states, however, that magnesium chloride can precipitate lecithin completely in an acetone-ether mixture, and we commenced experiments to test this. The work of Bloor [1929] came to our notice soon afterwards, and

[1] Methods of handling small quantities of liquids are described by Szent-Györgyi and Tominaga [1923], and we have found these helpful in designing our methods.

most of our work took the form of testing the modification of Nerking's method described by this author.

Estimation of phosphorus. Our estimations of phosphorus have been made by the colorimetric method of Briggs [1924].

The method was modified by keeping the quinol and the sodium sulphite in the same solution, 0·5 % quinol in 10 % sodium sulphite. 2 cc. of the solution are added for a total volume of 10 cc.

Extensive testing with inorganic phosphate solutions showed us that an accuracy of about 1 % can usually be obtained, when the amount of phosphorus is within some 30 % of that in the standard, which is best taken at 0·06 mg. P (as potassium dihydrogen phosphate). The Klett top-reading colorimeter, provided with a lamp, was used. Preliminary estimations are necessary when the amount of phosphorus to be estimated is quite unknown.

Estimations of organic phosphorus were made after ignition with sulphuric acid and hydrogen peroxide. The chloroform solution is taken to dryness in a 6-inch thick-walled test-tube in a stream of air, 0·3 cc. of concentrated sulphuric acid is added, followed by 0·5 cc. of Merck's perhydrol previously redistilled under low pressure. The ignition is carried out over a small flame, more peroxide being added as necessary, and the tube being rotated to rinse the walls towards the end of the ignition. After addition of water, the acid is neutralised approximately with sodium hydroxide.

Tests on the ignition of known amounts of inorganic phosphate gave complete recovery. With lipoid phosphorus the reproducibility was often within 1 %, and usually within 3 %.

Experiments with pure lecithin and magnesium chloride. We shall report experiments only in which the precipitation took place under the conditions used by Bloor, as the variations tried did not greatly affect the results.

From 1 to 3 cc. of an alcoholic solution of lecithin[1] was taken to dryness in a centrifuge-tube, and dissolved in 2 cc. of light petroleum and 7 cc. of acetone. Then was added 0·1 cc. of a saturated solution of hydrated magnesium chloride in absolute alcohol. After thorough stirring of the precipitate—precipitation of the salt takes place independently of the presence of lecithin—it was centrifuged down, the supernatant liquid poured off, and the precipitate washed with 2 cc. of acetone.

Phosphorus was estimated in the supernatant liquid and wash liquid; it was usually necessary to unite the liquids from several precipitations and to add some inorganic phosphate after ignition, in order to obtain enough phosphorus to estimate.

A "blank" correction was necessary; when no lecithin was used in the precipitation and washing, phosphorus was apparently present in the supernatant and wash liquids.

[1] The lecithin was made according to the method of Levene and Rolf [1927] by Dr H. I. Price, to whom we are indebted for it.

The figures obtained are given in Table I.

Table I. *Precipitation of lecithin by magnesium chloride.*

Mg. P taken	Mg. P not precipitated	Mg. P (blank)	Corrected mg. P not precipitated	% of P not precipitated
0·111	0·0086	0·0022	0·0064	6
0·333	0·0231	,,	0·0209	6
0·114	0·0099	,,	0·0077	7
0·114	0·0096	,,	0·0074	7
0·342	0·0435	,,	0·0413	12
0·113	0·0080	,,	0·0058	5
0·113	0·0108	,,	0·0086	7
0·067*	0·0045	0·0026	0·0019	3
0·067*	0·0047	,,	0·0021	3
0·134*	0·0085	,,	0·0059	4
0·134*	0·0058	,,	0·0032	2
0·067*	0·0037	0·0024	0·0013	2
0·067*	0·0039	,,	0·0015	2

* For these estimations 200 mg. of lecithin were precipitated with $MgCl_2$ and washed as in the micro-estimations. The precipitate was extracted with ether and chloroform, and the extract finally dissolved in chloroform together with a little alcohol.

Evidently about 93 % of the lecithin is precipitated, or about 97 % when the lecithin has been purified.

Experiments were then made to test whether the reason for incomplete precipitation was solubility of the lecithin in the precipitant mixture. Magnesium chloride precipitates, after the usual washing, were washed again with a mixture of 7 cc. of acetone and 2 cc. of light petroleum, and phosphorus estimated in this liquid. When 0·113 mg. of lecithin-P was taken initially, and two washings made, the uncorrected P from two washings was about 0·0061 mg. as a mean. Subsequent pairs of washings gave about the same value. The value suggests that most of the lecithin that remains unprecipitated does so on account of its solubility.

According to Bloor the lecithin may be extracted from the magnesium chloride precipitate with moist ether, solution being, however, a slow process. The water in the ether dissolves the magnesium chloride to form a separate phase.

We have found it very difficult to extract all the lecithin with ether. It does not dissolve completely, but can be decanted suspended in ether. From three to five washings with ether are necessary. The recoveries of phosphorus were then 88, 96, 91 and 89 % of that taken. These values are moderately satisfactory, but the procedure is too laborious to be a practical method.

Extraction with chloroform was then tried: the solvent was brought to boiling-point and extraction made three or four times. This is a simpler method, but the recovery is variable. The percentage recoveries found were 88, 86, 85, 96, 94, 89, 88, 83, 75, 92 and 78.

We have also tried estimating the phosphorus in the magnesium chloride precipitate by igniting this directly. The recovery is usually from 95 to 102 %, but the ignition in presence of magnesium chloride is lengthy and laborious.

Our conclusion is that the most satisfactory way of using the magnesium chloride method is to estimate total phosphorus and subtract from it phos-

phorus not precipitated by magnesium chloride. This variant has been used in the work of the following section.

Experiments with lipoid mixtures and magnesium chloride.

The magnesium chloride precipitation has been made on tissue extracts, containing phosphatides, cholesterol and fats.

The data obtained are given in Table II. "Blank" corrections were not applied, and if the behaviour were the same as it is for pure lecithin about 5 % of the phosphorus would have remained unprecipitated.

Table II shows that in some cases the percentage not precipitated is much higher. It is highest when the fat content of the extract is high (shown by a high value of the ratio of total lipoids to total phosphorus).

Table II. *Precipitation in lipoid mixtures.*

Tissue extract	$\frac{\text{Total lipoids}}{\text{Total P}}$	% P not precipitated
2 A	125	26·0
2 B	317	20·0
2 C	343	18·5
6 B	175	15·2
5 B	43	11·3
5 A	50	9·3
7 B	41	8·6
3 A	48	5·9
5 C	33	5·6

Now there is no special reason to believe that, in the presence of fat, alcohol will extract from tissues non-phosphatide-phosphorus that would otherwise remain unextracted.

The most probable conclusion is that fat interferes with the precipitation of phosphatides by magnesium chloride.

If this is so, Bloor's method for estimating phosphatides will tend to give low results, quite apart from the fact that the ether extraction in our hands has not given complete recovery.

Conclusion. We have been forced to assume that the whole of the phosphorus in our extracts represents phosphatides. The assumption is probably not much in error, if we may judge from a comparison between the results of the magnesium chloride method on pure lecithin and on tissue extracts containing little fat. This comparison confirms the general impression that anhydrous solvents extract little phosphorus-containing material other than phosphatides.

We have calculated the phosphatide content of our extracts on the basis that the phosphorus content of phosphatides is 4·00 %. This figure may be a little too high—Levene and Rolf [1927] find about 3·9 % for pure lecithin, and Levene and West [1916] about 3·7 % for kephalin—but the error introduced should tend to offset any error in assuming that all the phosphorus found represents phosphatide.

The phosphorus percentage will vary somewhat in phosphatides from

different tissues, but the available data suggest that the variation is not great. The objections that have been raised to assuming a constant composition seem to have been over-emphasised.

The estimation of cholesterol.

The gravimetric method, in which cholesterol is combined with digitonin, has been used.

The compound has been precipitated in a manner similar to that employed by Szent-Györgyi [1923]. A quantity of material containing from 0·5 to 3 mg. of cholesterol is taken to dryness in a 10 cc. centrifuge-tube, dissolved in 2 cc. of acetone, and 1 cc. of digitonin solution added (1 g. of commercial digitonin dissolved in 50 cc. of 80 % alcohol). After being stirred and warmed, the liquid is evaporated to about one-half in a stream of air. After about 15 minutes the precipitate is washed in the centrifuge-tube, in a manner not very different from that of Tominaga [1925], twice with 7 cc. of ether, and the supernatant liquid poured off into the filter-tube. Three washings are then given in the heat with 7 cc. (each time) of a mixture containing 80 % of acetone and 20 % of water. Of the wash-liquids it is only the 80 % acetone that removes much of the excess digitonin.

The precipitate is then washed on to the filter with about 10 cc. of pure acetone, followed by 6 cc. of ether. The filter-tube is then left in an electrically heated oven at 105–110° for an hour. On removal it is capped and left in a calcium chloride desiccator for 20 minutes, after which it is weighed.

Experiments were first made with Jena sintered glass filter-tubes. Unfortunately the filters with the finest pores (G 4) often became clogged with the precipitate, while those with larger pores (G 3) allowed the precipitate to pass through to some extent. The use of sintered glass filters was therefore abandoned.

The type of filter-tube described by Szent-Györgyi [1923] was employed, but lighter caps were used which were flat at the top and had somewhat flattened sides. The filter-tubes weighed, complete, about 5 to 6 g. The asbestos used was Powminco Grade xx; a little solid asbestos was followed by an aqueous suspension, and stronger suction succeeded gentle suction. After washing with water, acetone and ether the tubes were heated and then weighed; this procedure was repeated until the weight became approximately constant. The weight usually becomes constant within 0·04 mg., but further washing would result in further loss of weight.

Similarly when cholesterol digitonide is on the filter, continued washing leads to a larger and continued loss of weight. Any method of using the filter-tubes has to be established empirically.

To test the method described above a series of estimations was made on pure cholesterol[1]. A solution in absolute alcohol containing 1·021 mg. per cc.

[1] We are indebted for the cholesterol to Mr W. Doran, Department of Organic Chemistry, University of Liverpool.

was used to obtain the data in Table III. The average deviation of the estimations from the "theoretical" value (taking the precipitate to contain 25 % of cholesterol) is about 3 %. Tominaga claimed an accuracy within 1 %. Probably we could have obtained greater accuracy if every filter had been prepared with extreme care, many being rejected, and if those accepted had been used for only a few estimations. We have used a filter about eight times before renewing the packing.

Table III. *Weight of digitonide (mg.) from cholesterol.*

	1 cc.	2 cc.	3 cc.
	4·04	8·50	11·95
	3·91	8·66	12·58
	3·96	8·12	—
	4·11	7·94	—
	3·87	—	—
Mean	3·98	8·30	12·26
Theoretical	4·08	8·17	12·25
Average percentage deviation from theoretical	2·8	3·4	2·6

Estimations on tissue extracts. Although several papers have been published on estimation of small amounts of cholesterol by the gravimetric method, and the methods were apparently designed for estimating cholesterol in tissue extracts, the method seems to have been little applied to that purpose.

Our experience of the application of the method has not been very satisfactory.

Free cholesterol. When much fat in relation to cholesterol is present, the precipitate is contaminated with fat. We have not judged it wise to remove, prior to the estimation, the fat which often precipitates on cooling a hot acetone solution, as this may contain cholesterol. The fat is removed by washing the precipitate as described, removal being most rapid in the heat.

An examination of the results of a series in which two estimations were made on each of a number of tissue extracts showed that the presence of much fat is associated with less accurate agreement between the results. When the ratio of total lipoids to cholesterol was over 20 (averaging about 50), the average difference between the two values was 8·7 %. With lower values for the ratio (about 8–12), the average difference between the two values was only 4·3 %. Evidently the presence of much fat leads to irregular values.

When several estimations on the same tissue extract have been made with different quantities, there is a certain tendency for lower relative values to be found, the more the material taken for an estimation. This appears to indicate that fat tends to inhibit formation of a digitonide precipitate that is retained by the filter.

Total cholesterol. A portion of the tissue extract is taken to dryness, and then there is added to it 1 cc. of ether followed by 1 cc. of 15 % sodium ethoxide in absolute alcohol. After being warmed to dissolve the lipoid material, the solution is allowed to stand at least 24 hours. According to

Gardner and Fox [1924], hydrolysis of cholesterol esters should then be complete. The solution is taken down to dryness, and 2 cc. of 2*N* sulphuric acid are added. After shaking, the material is extracted four times with ether in the test-tube used. The ether extract is evaporated to dryness.

Estimation of cholesterol is then carried out in the same way as for free cholesterol, except that the extract has been dissolved in 2 cc. of ether instead of acetone. Ether was used instead of acetone with a view to lessening the interference of fatty material with the precipitation, as most of the fat then stays in solution. The precipitate of digitonide is more flocculent with ether.

Preliminary estimations, made to test whether the use of ether gives the same results as acetone on free cholesterol, are given in Table IV. The data also serve to show the kind of reproducibility the methods give. The acetone and ether methods give very similar results, as far as the data go. Further tests would be worth while.

Table IV. *Comparison of acetone and ether methods on free cholesterol.*

	mg. precipitate acetone method	mg. precipitate ether method
Pure cholesterol	12·79	12·56 12·67
Tissue extract containing much fat	6·87 6·64	7·03 7·09
Tissue extract containing little fat	8·16 8·01	7·50 7·82
" "	8·52 8·84	8·78
" "	12·94 13·47	13·42 12·90

An examination of results, similar to that made in the case of free cholesterol determinations, showed that the extracts containing much fat did not tend to give more irregular results than the others.

The series shows an average difference of 5·4 % between the results of the two estimations on each extract; for the free cholesterol series the corresponding figure was 5·8 %. When the estimations on pure cholesterol in Table III are taken in pairs the average difference between the results for the two members of each pair is 4·0 %. The mean result of each pair of estimations diverges on the average from the theoretical value by 1·8 %, or by half the previous figure.

The values obtained from the pairs of estimations on free and bound cholesterol in tissues should therefore be on the average less than 3 % from the true value, apart from the existence of a systematic error, such as a tendency of fat to produce low values. It must be pointed out, however, that further repetitions of some of the estimations sometimes show much larger discrepancies.

Comparison with other work. Osato and Heki [1930] have made microgravimetric estimations of cholesterol in tissues, but do not yet appear to have published details of their method. The only other work on this subject

we are aware of is that of Onizawa [1928], who extracted cholesterol from tissues by a modification of Fox's method, thereby removing fats. His results on pure cholesterol and on tissues are apparently more reproducible than ours. The only apparent difference in the estimation of cholesterol is that this author washed his precipitates finally with water and dried them in a vacuum oven, as did Szent-Györgyi. Our results suggest that removal of fat prior to the estimation should lead to greater reproducibility.

SUMMARY.

Methods are described for the extraction and estimation of phosphatides and cholesterol in 5–20 g. of tissue.

Chloroform does not extract lipoid substances completely from dried tissues. Magnesium chloride precipitates pure lecithin almost completely, but the presence of much fat appears to interfere with precipitation of phosphatides. The analytical method described by Bloor [1929] probably gives low results. The phosphorus in our extracts is probably almost entirely derived from phosphatides.

The micro-gravimetric method has been tested on pure cholesterol and applied to tissue extracts. Much fat lessens the accuracy of free cholesterol determinations, but in the presence of ether does not appear to interfere with estimations of total cholesterol.

In conclusion the authors wish to acknowledge their indebtedness to Prof. W. C. M. Lewis for his interest in this work, and to Prof. H. J. Channon for some helpful advice.

REFERENCES.

Bell (1925). *Lancet*, ii, 1003.
—— (1930). Some aspects of the cancer problem. (London.)
Bloor (1929). *J. Biol. Chem.* **82**, 273.
Briggs (1924). *J. Biol. Chem.* **59**, 255.
Bullock and Cramer (1913–14). *Proc. Roy. Soc. Lond.* B **87**, 236.
Gardner and Fox (1924). *Biochem. J.* **18**, 1058.
Jowett (1931). *Biochem. J.* **25**, 1991.
Levene and Rolf (1927). *J. Biol. Chem.* **72**, 587.
—— and West (1916). *J. Biol. Chem.* **24**, 41, 111.
Lewis (1927). *J. Cancer Research*, **11**, 16.
Nekludov and Chalatov (1929). *Biochem. Z.* **208**, 60.
Nerking (1910). *Biochem. Z.* **23**, 262.
Onizawa (1928). *J. Biochem. (Japan)*, **10**, 45.
Osato and Heki (1930). *J. Biol. Chem.* **87**, 541.
Smith and Mair (1911). *J. Path. Bact.* **16**, 130.
—— —— (1913). *J. Path. Bact.* **17**, 609.
Szent-Györgyi (1923). *Biochem. Z.* **136**, 102, 107.
—— and Tominaga (1923). *Biochem. Z.* **146**, 226.
Tominaga (1925). *Biochem. Z.* **155**, 119.

CCXV. THE PHOSPHATIDE AND CHOLESTEROL CONTENTS OF NORMAL AND MALIGNANT HUMAN TISSUES[1].

By MAURICE JOWETT.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received October 24th, 1931.*)

PHOSPHATIDES and cholesterol are invariably constituents of animal tissues. They play a part in fat metabolism [MacLean and MacLean, 1927], and may be to some extent storage materials, but the work chiefly of Mayer and Terroine has shown that this is true only to a limited extent, and that they tend to maintain constant their quantities in any one tissue.

It is natural, then, that a rôle in regulating protoplasmic structure and function has been attributed to them. Of the modes of approach only one will be discussed here, which is derived from the facts that lecithin and cholesterol lower the interfacial tension between oil and water [Corran and Lewis, 1924; Okunev, 1928], and are emulsifying agents. Lecithin favours a dispersion of oil in water and cholesterol a dispersion of water in oil, the two being in this sense antagonistic, as shown by Corran and Lewis.

Similar action may well take place in the cytoplasm, particularly in cell-membranes. Corran and Lewis have expressed the view that a cell of which the contents show a high lecithin-cholesterol ratio is likely to be more permeable to water-soluble substances than a cell with a low ratio. This view receives some support from the fact that lecithin (or substances derived from it) favours haemolysis and cholesterol inhibits it *in vitro*. Too much lecithin presumably makes the membrane too labile for continued existence, while cholesterol counteracts the effect. Such effects may also be the reason for the retarding action of lecithin and the accelerating action of cholesterol on the growth of animal tumours found by Robertson and Burnett [1913], Moravek [1927] and Rondoni [1930].

The lipin content of a cell may, on the view of Corran and Lewis, influence its supply of building materials and metabolites.

This may permanently affect its general activities. In this way variation in lipin content might be one of many causal factors in the varied cell-differentiation that takes place in embryonic development, and might remain

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell, University of Liverpool).

in the adult cell as an expression of the variation in development. Cells having in some respects higher metabolism or more complex structure would be expected to have the higher lipin ratio.

An alternative view can be suggested on functional grounds. In the resting cell permeability to water-soluble substances may not be a limiting factor in maintenance of structure and metabolism. The growing cell, or more active resting cell, may require greater permeability to obtain its supplies. Hence the growing cell, or resting cell of high metabolism, may show a high lecithin-cholesterol ratio.

Yet little evidence can be offered that insufficient permeability hinders the mammalian cell from obtaining supplies.

The possible consequences of the antagonistic emulsifying properties can be developed in another way, not involving permeability. Although lipins probably tend to concentrate in cell-membranes, most of the material will usually remain in the interior of the cell, and may there share in regulating the state of aggregation of protoplasm. Lecithin is likely to favour fine dispersion in their aqueous medium of the protein-lipin colloids of protoplasm, and cholesterol is likely to tend to aggregate them. Finer dispersion will probably be accompanied by greater hydration. It may thus be argued that a high lecithin-cholesterol ratio will be found in cells of high water-content, high lability and high enzymic activity—since fine dispersion gives more available internal surface. These characteristics in a general way are those of young and growing cells, which have a high water-content and a high metabolic activity, and which must possess a labile structure to be able to undergo the rearrangements that precede cell-division.

Some evidence can be put forward in favour of the rôles attributed above to lipins. From a general colloid-chemical point of view their peptising effects are not likely to be restricted to oil-water systems, and work such as that of Handovsky, Lohmann and Bosse [1925] and of Theorell [1930] shows that there is very close association between lipins and serum or plasma proteins; Theorell finds that the less easily salted out the protein is, the higher is the phosphatide-cholesterol ratio of the associated lipin. Likewise, *in vitro* they affect the activity of enzyme extracts[1].

In the present paper are presented analyses of human tissues which, as far as they go, and in conjunction with already published data, tend to confirm the view that growing cells, in particular malignant cells, have a high phosphatide-cholesterol ratio. This is a finding not restricted to such cells; and there are other findings that theory does not predict. No exact and simple confirmation can be expected: factors other than lipins are concerned in

[1] Tsuneyoshi [1927] finds that lecithin increases and cholesterol diminishes (1) the consumption of oxygen by tissue powder in presence of succinic acid, and (2) the oxidation of glycine on charcoal. Eichholtz [1924] finds that lecithin promotes catalase action in serum. Cholesterol retards peptic action [Dörle, 1923]. However lecithin sometimes retards enzymic activity, as in the case of tryptic digestion [Hagihara, 1924; Standenath, 1925].

growth and metabolism. In addition, the material analysed seldom contains only one type of cell; our methods do not distinguish lecithin from other phosphatides, which may have quite different surface effects; nor is the capillary effect of cholesterol esters clear.

Experimental.

The tissues examined were removed at operations in Liverpool. The author is indebted for them principally to Prof. W. Blair Bell, Mr M. M. Datnow and Miss G. Griffith. Histological examinations of representative pieces of each tissue were made by Mr M. M. Datnow.

To avoid chemical change, tissues were minced and immersed in alcohol or acetone as soon as possible; this was complete in from 1 to 3 hours after excision for all but five specimens, which were kept overnight in a refrigerator.

The analytical methods have been described in an accompanying paper [Jowett and Lawson, 1931]. Mr E. W. Lawson assisted in the analytical work. Estimations were carried out in duplicate (with the exception of estimation of dry weight), and frequently several times when agreement was not good.

Tissues were sometimes kept some time in alcohol, and extracts were usually kept in chloroform for a considerable time, before the analyses were complete. Serious changes are unlikely to occur under these conditions.

Discussion of analytical results.

The analytical data that have been obtained are given in Table I. The tissues are seldom composed almost entirely of one type of cell. Some of the malignant tissues are diluted with fibrous tissue; one specimen of malignant tissue and some of the muscular tissues are diluted with fat[1]. Comparisons are made more difficult, but a number of conclusions can nevertheless be drawn.

According to many workers, for instance Cramer [1916], the water-content of young and of malignant tissues is higher than that of resting tissues, but in the present data this tendency seems usually to be obscured for malignant tissue.

In the present work, malignant tissues and normal epithelial tissues show on the whole higher contents of phosphatides and of cholesterol than do fibromyomata and normal muscle.

Of the seven specimens of malignant tissue, those containing the highest proportion of malignant cells have definitely the highest content of phosphatides and cholesterol, and tend to show the highest ratio of phosphatides to free cholesterol.

The data for cholesterol show a number of discrepancies, free cholesterol being sometimes found higher than total cholesterol, but nevertheless the conclusion may be drawn fairly definitely that of the tissues analysed malignant

[1] In Table I (last column), fat has been calculated by subtracting from the total lipoids the phosphatides and cholesterol; the figure will include fatty acids bound to cholesterol.

tissues are the only class in which the proportion of cholesterol in the ester form is considerable, averaging nearly 40 %.

Chorionic villi, according to Bell [1930], share many of the properties of malignant tissue. Of the two specimens analysed, the one obtained at an early period of pregnancy and containing a large proportion of syncytium should show these characteristics more than that at a later period, which

Table I.

No.	Tissue	% dry weight	% on dry weight of: Total lipoids	Phosphatides	Cholesterol: Free	Cholesterol: Total	Phosphatides / Free cholesterol	% cholesterol as esters	% fat in total lipoids
	Malignant:								
11 A	Carc. kidney (all malignant)	22·3	29·89	11·70	1·238	2·026	9·45	39	54
15 A	Sarc. uterus (all malignant)	17·5	19·51	8·98	1·667	2·64	5·4	37	40
3 A	Carc. omentum (¾ growth)	18·65	17·42	9·12	1·545	2·23	5·9	31	35
2 A	Carc. breast (40 % growth)	20·7	27·95	5·58	0·827	1·389	6·75	40	75
8	Carc. caecum (half fibrous tissue)	15·15	13·46	5·51	1·113	1·759	4·95	36	46
9 A	Carc. breast (half fibrous tissue)	20·1	14·71	5·36	1·138	2·096	4·7	46	50
14 A	Carcinoma (growth ¼, blood clot ¾)	12·5	9·22	4·06	1·053	1·208	3·9	13	43
	Fibromyomata uteri:								
5 B	All healthy growth	21·0	4·66	2·71	0·544	0·619	5·0	12	29
10 A	All healthy growth	24·5	3·38	1·395	0·320	0·286	4·4	?	50
7 A	Hyaline degeneration of half growth	19·5	2·84	1·45	0·247	0·344	5·9	28	37
16 B	Healthy growth, but chiefly muscle	20·5	7·48	5·93	0·753	0·605	7·9	?	13
	Epithelial tissues:								
5 C	Normal ovary	19·0	11·04	8·25	0·727	0·835	11·35	13	17
11 B	Normal kidney	20·2	17·58	8·04	1·522	1·587	5·3	4	45
17	Chorionic villi, 10–12 weeks	11·94	16·33	8·24	1·407	1·492	5·8	6	40
13	Chorionic villi, 7 months	15·5	10·90	5·10	1·133	1·101	4·5	0	43
	Uterine muscle (healthy):								
5 A	—	21·2	6·55	3·30	0·749	0·851	4·4	12	37
7 B	With a little mucosa	19·3	4·97	3·04	0·606	0·636	5·0	5	36
10 B	—	19·7	7·38	2·97	0·818	0·595	3·6	?	51
15 B	With a little mucosa	21·4	8·65	3·77	0·788	—	4·8	—	45
16 A	—	18·2	8·38	5·42	0·936	0·935	5·8	0	24
	Muscle:								
2 B	Pectoral	25·9	33·74	2·66	0·537	0·717	4·95	25	90
2 C	Pectoral	24·9	29·24	2·13	0·615	0·901	3·5	33	90
9 B	Striated, pectoral	27·1	25·19	2·84	0·291	0·242	9·8	?	88
14 B	Leg	15·8	11·03	5·74	1·042	0·843	5·5	?	40
14 C	Leg	22·5	7·38	2·83	0·264	0·235	10·7	?	59
6 B	Bowel muscle, ⅛ mucosa	17·1	23·85	3·40	0·660	0·628	5·15	?	83

contains little syncytium. Actually the younger has a greater water-content, a higher lipin content and a higher phosphatide-cholesterol ratio. Neither specimen contains an appreciable amount of cholesterol esters[1].

Associated with growing tissues we therefore appear to have a high lipin content and a high phosphatide-cholesterol ratio, but these are not restricted to such tissues. In the present data normal ovary[2] and normal kidney have

[1] Bienenfeld [1912] found a large proportion of bound cholesterol in early placentae, but her extraction appears to have been incomplete.

[2] The tissue analysed contained approximately: lutein tissue 2 parts, corpora albicantes 4, and ovarian stroma 11.

a high lipin content; ovary and two specimens of muscle have a very high phosphatide-cholesterol ratio.

That these quantities have nevertheless a definite significance for growing tissue of an abnormal nature can be shown in another way, by comparing abnormal tissue with the neighbouring tissue from which it may have arisen. Theoretically, the comparison should probably be made between the abnormal cell and the type of normal cell from which it arose. Practically, we are restricted to a comparison with neighbouring tissue of the same general type as that from which the abnormal may have arisen. Comparisons of this kind are given in Table II.

Table II. *Comparisons between growths and normal tissue.*

No.	Tissue	% on dry weight: Phosphatides	% on dry weight: Free cholesterol	Phosphatides / Free cholesterol	% cholesterol bound	% fat in total lipoids
11 A	Carc. kidney	11·70	1·238	9·45	39	54
11 B	Normal kidney	8·04	1·522	5·3	4	45
15 A	Sarc. uterus	8·98	1·667	5·4	37	40
15 B	Uterine muscle	3·77	0·788	4·8	—	45
5 B	Uterine fibromyoma	2·71	0·544	5·0	12	28
5 A	Uterine muscle	3·30	0·749	4·4	12	37
7 A	Uterine fibromyoma	1·45	0·247	5·9	28	37
7 B	Uterine muscle	3·04	0·606	5·0	5	36
10 A	Uterine fibromyoma	1·395	0·320	4·4	?	50
10 B	Uterine muscle	2·97	0·818	3·6	?	51
16 B	Uterine fibromyoma	5·93	0·753	7·9	?	13
16 A	Uterine muscle	5·42	0·936	5·8	0	24

The two malignant tissues are fortunately not admixed with normal tissue; of the four fibromyomata, one is degenerating and one is admixed with normal muscle. In all cases the abnormal tissue shows a higher ratio of phosphatide to free cholesterol than the normal tissue. Malignant tissue shows also a higher phosphatide content than the normal, but for the innocent growths the converse is usually true for both phosphatides and cholesterol. If the data for the fibromyomata and corresponding normal tissues be examined, it will be seen that regularities, which may be extended to the proportion of free fat in the lipoids, emerge only if each fibromyoma is compared with the neighbouring normal muscle removed at the same operation. If fibromyomata and uterine muscle are compared indiscriminately as classes, regularities are much less evident.

This suggests tentatively that abnormal tissue retains much of the character of the normal tissue it arises from; a suggestion in agreement with the well-known pathological fact.

In two cases we have data that may be used to answer in a preliminary way the question whether the composition of normal tissue situated close to a malignant growth differs from the composition of that situated further away. The data are given in Table III. In one case a carcinoma of the breast (2 A)[1]

[1] This specimen contained: healthy growth 6, fibrous tissue 4, leucocytes and necrotic tissue 3, and fat 2 parts.

is imbedded in pectoral muscle, and that near the growth (2 B) may be compared with that situated further away (2 C). In the other case a carcinoma (14 A) is surrounded by leg muscle, neighbouring (14 B) or further away (14 C). The specimens of muscle show no gross difference histologically, but

Table III. *Composition of tissue near a growth.*

	% on dry weight			Phosphatides / Free cholesterol
No.	Phosphatides	Free cholesterol	Total cholesterol	
2 A	5·58	0·827	1·389	6·75
2 B	2·66	0·537	0·717	4·95
2 C	2·13	0·615	0·901	3·5
14 A	4·06	1·053	1·208	3·9
14 B	5·74	1·042	0·843	5·5
14 C	2·83	0·264	0·235	10·7

examination of the figures shows some tendency for the normal muscle close to the growth to be more like the growth in composition than is the more distant muscle. Little more can be said with the limited data available, but the subject merits further investigation.

Previous work.

The results of previous work, some of it carried out by methods of doubtful value, will be briefly considered in relation to our findings.

The phosphatide contents of tissues tend to be higher in malignant tissues than in normal, in nervous tissue and glands than in muscle, and in young tissues than in old. In general, active tissues show higher contents than inactive. We may cite, for instance, the work of Enselme and Enselme [1927], who find more phosphatides in the malignant breast than in the normal gland. Decrease of phosphatide content on passing from young to older tissues is found for the testicle by Koch and Woods [1905], for the bone marrow by Glikin [1907], and the carcase of the young white rat by Sinclair [1930]. Sorg [1929] finds that in three kinds of rabbit muscle, the phosphatide content is greater in muscle of greater endurance capacity. According to Momigliano [1925], during involution of the human corpus luteum the phosphatide content decreases. Data are quoted by Bloor, Okey and Corner [1930] to show that the variation of phosphatide content in different animals parallels the intensity of metabolism; the content is, for instance, higher in the mouse than in man.

Bürger and Schlomka [1928] find that the cholesterol content of human skin decreases with increasing age, and Dam [1931] finds a decrease with age for the newly hatched chick. Burgheim and Joel [1931] find the cholesterol content of malignant growths greater than that of benign growths.

The ratio of phosphatides to cholesterol was found to be higher in a mouse tumour of rapid growth than in one of slow growth by Bullock and Cramer [1913–14]. In the corpus luteum of the sow, according to Bloor, Okey and Corner [1930], the phosphatide content parallels the physiological activity,

while the free cholesterol changes similarly but to a less extent. According to Iwano [1925] the lecithin-cholesterol ratio falls with age in transplanted rat tumours.

It cannot be pretended that indiscriminate comparison of different tissues shows any intelligible relations between lipin contents or lipin ratios and any individual structure or function as yet compared. The data of Osato and Heki [1930] on rabbit tissues may be used to illustrate the point. Their mean figures yield the following ratio of phosphatides to free cholesterol: heart muscle 21, liver 16, skeletal muscle 10·5, spleen 9·1, kidney 8·6, lung 6·7, and blood 3·8. It is understandable that heart muscle shows a higher value than skeletal muscle, but further interpretation is at present impossible. Muscle, liver and kidney have all different chemical functions, and a common factor showing a relation with the lipin ratios is yet to be found.

The relation of cholesterol esters to growth is not yet clear. The author finds much of the cholesterol in malignant tissue to be bound; but in transplanted tumours of mice and rats, according to Bullock and Cramer [1913–14], Iwano [1925], and Bolaffi [1929] there is little bound cholesterol. In placenta, according to the present work, there is little bound cholesterol, while Dam [1929, 1931] finds a large proportion in newly hatched chicks, which decreases in the first few weeks of life. Cholesterol esters accompany regression in the corpus luteum [Bloor, Okey and Corner, 1930; Momigliano, 1925]. However, Onizawa [1929] finds that the bound cholesterol varies more than the free cholesterol in tissues, and other data suggest the same. The conclusion may be due to greater experimental error, but yet it seems likely that cholesterol esters are fairly inert and constitute storage material.

Summary.

A discussion of the physico-chemical properties of lecithin and cholesterol leads to the conclusion that the lecithin-cholesterol ratio of growing cells, and probably other active cells, should be high.

A limited set of analyses of human tissues gives the following results. Pure malignant tissues have a higher phosphatide and cholesterol content, and tend to show a higher phosphatide-cholesterol ratio, than do malignant tissues admixed with normal tissue. Malignant tissues show a high proportion of bound cholesterol. Malignant and benign tumours show a higher phosphatide-cholesterol ratio than do the neighbouring tissues from which they may have arisen. Previous work has shown a definite parallelism in tissues between growth and activity and the phosphatide content.

In conclusion, the author wishes to thank Prof. W. C. M. Lewis for the interest he has taken in this work.

REFERENCES.

Bell (1930). Some aspects of the cancer problem. (London.
Bienenfeld (1912). *Biochem. Z.* **43**, 245.
Bloor, Okey and Corner (1930). *J. Biol. Chem.* **86**, 291.
Bolaffi (1929). *Z. Krebsforsch.* **30**, 170.
Bullock and Cramer (1913–14). *Proc. Roy. Soc. Lond.* B **87**, 236.
Bürger and Schlomka (1928). *Z. ges. exp. Med.* **63**, 105.
Burgheim and Joel (1931). *Klin. Woch.* **10**, 397.
Corran and Lewis (1924). *Biochem. J.* **18**, 1364.
Cramer (1916). *J. Physiol.* **50**, 322.
Dam (1929). *Biochem. Z.* **215**, 468.
—— (1931). *Biochem. Z.* **232**, 269.
Dörle (1923). *Z. ges. exp. Med.* **34**, 406.
Eichholtz (1924). *Biochem. Z.* **151**, 187.
Enselme and Enselme (1927). *Bull. Soc. Chim. Biol.* **9**, 1017.
Glikin (1907). *Biochem. Z.* **4**, 235.
Hagihara (1924). *Biochem. Z.* **144**, 482.
Handovsky, Lohmann and Bosse (1925). *Pflüger's Arch.* **210**, 63.
Iwano (1925). *J. Biochem.* (*Japan*), **4**, 481.
Jowett and Lawson (1931). *Biochem. J.* **25**, 1981.
Koch and Woods (1905). *J. Biol. Chem.* **1**, 203.
MacLean and MacLean (1927). Lecithin and allied substances. (Longmans, Green and Co., London.)
Momigliano (1925). *Z. Gynäkol.* **49**, 684.
Moravek (1927). *Arch. Clin. Cancer Research*, **3**, 99.
Okunev (1928). *Biochem. Z.* **198**, 296.
Onizawa (1929). *J. Biochem.* (*Japan*), **10**, 409.
Osato and Heki (1930). *J. Biol. Chem.* **87**, 541.
Robertson and Burnett (1913). *J. Exp. Med.* **17**, 344.
Rondoni (1930). *Z. Krebsforsch.* **32**, 416.
Sinclair (1930). *J. Biol. Chem.* **88**, 575.
Sorg (1929). *Z. physiol. Chem.* **182**, 97.
Standenath (1925). *Biochem. Z.* **155**, 245.
Theorell (1930). *Biochem. Z.* **223**, 1.
Tsuneyoshi (1927). *J. Biochem.* (*Japan*), **7**, 235.

CCXVI. THE LOSS OF GLUCOSE FROM DRIED PEAS ON SOAKING.

By WINIFRED MARY CLIFFORD.

From the Physiology Department, King's College of Household and Social Science, Campden Hill Road, Kensington, W. 8.

(*Received October 22nd, 1931.*)

The common treatment of dried peas when used as human food is to subject them to a prolonged soaking so that they may imbibe water and to follow this up by boiling the peas.

Hutchison [1927] says of this process, "...soaking (is) inevitably accompanied by some loss of protein and mineral matter and also of carbohydrates." Similar statements are met with in many cookery books and have given rise to the idea that such a loss is considerable and may cause a decided drop in the food value of the peas.

Masters [1918] investigated the loss incurred by beans on soaking and found that 100 g. soaked in 250 cc. tap or distilled water lost 1·5–2·2 % solids and of this a considerable proportion consisted of nitrogenous extractives and salts. It was decided to carry out similar experiments with peas with regard to the loss of carbohydrate.

At the same time, opportunity was given to investigate the further statement of Hutchison [1927], that "peas do not readily soften in water containing lime...hence the importance of adding soda to hard water in which pulses are to be soaked."

Experimental methods.

The loss of carbohydrate from peas when soaked in various solutions was determined as follows.

100 g. of dried peas were placed in 250 cc. of the liquid and kept overnight. In the morning the surplus was strained off through butter-muslin and measured. This fluid, if acid, was neutralised with anhydrous sodium carbonate and estimations of reducing sugar carried out, using Benedict's method. An osazone was made from the specimen in any series giving the strongest reaction, and a heavy crop of glucosazone crystals resulted in every case. Therefore all reducing substances were calculated as glucose. No iodine reaction was obtained with any fluid investigated, and there were only minute traces of any non-reducing sugar giving rise to reducing sugars on hydrolysis. Each fluid gave faint reactions with the protein colour tests, but no differences

were noticed in sugar titrations when carried out before or after removal of the traces of protein.

For experiments on the rate of cooking, the peas when strained from their soaking fluids were loosely tied in butter-muslin and immersed in an open saucepan of boiling water. This water was kept boiling vigorously for 40 minutes when the peas were removed. Peas soaked in distilled water were invariably well cooked in this time, and the others were compared with them.

Experimental results.

A = cc. fluid remaining after soaking (originally 250 cc.).
B = g. glucose in A, *i.e.* glucose lost from 100 g. peas.

Inorganic acids.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
HCl	147	0·13	147	0·25	148	0·24	150	0·24	151	0·13	154	0·17	153	0·62
HNO_3	145	0·11	147	0·19	149	0·18	150	0·17	152	0·11	153	0·12	152	0·38
H_3BO_3	144	0·16	145	0·15	145	0·15	145	0·15	145	0·13	—	—	—	—
H_2SO_4	144	0·11	145	0·18	147	0·21	150	0·21	152	0·14	152	0·13	152	0·21
H_3PO_4	135	0·16	138	0·20	140	0·30	143	0·45	146	0·52	148	0·53	150	0·30

Organic acids.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.
Acid	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Formic	146	0·12	148	0·41	152	0·54	154	0·60	154	0·60	154	0·59	154	0·43
Acetic	140	0·13	141	0·28	144	0·43	148	0·76	150	0·78	155	0·80	154	0·80
Lactic	143	0·10	144	0·37	144	0·47	150	0·49	152	0·49	158	0·50	160	0·47
Oxalic	134	0·12	140	0·35	142	0·39	148	0·45	150	0·45	150	0·23	154	0·14
Malic	140	0·13	142	0·18	142	0·21	144	0·32	146	0·44	150	0·51	154	0·55
Tartaric	140	0·11	144	0·22	144	0·28	143	0·35	148	0·42	150	0·45	150	0·46
Citric	138	0·12	138	0·18	138	0·25	138	0·31	142	0·38	148	0·45	148	0·42

Of the inorganic acids boric acid alone appears to have no effect. The results with hydrochloric, nitric and sulphuric acids are similar to each other. There is a decrease of water taken up by the peas with increased strength of acid. The sugar lost does not run parallel with this change in absorption, neither does it vary directly with strength of acid. With 0·5 *N* acids most sugar is lost, but with 0·125 *N* only as much as with distilled water. There is another peak of relatively high sugar loss at 0·015 *N*–0·06 *N*. These results suggest that, though with the strongest acid used, hydrolysis of a complex carbohydrate may account for the increased glucose loss, in the lower concentrations some other factor is responsible for the result. This factor is not a simple increase in permeability of the plant cells since with distilled water there is a maximum uptake of water with a minimum of sugar loss.

Phosphoric acid differs from the other inorganic acids in that more sugar is lost from the peas on soaking. This loss is greatest from 0·06 *N* to 0·25 *N* and is considerably decreased at 0·5 *N*. As with the other inorganic acids the amount of liquid taken up by the peas decreases steadily with increasing

strength of acid. The curve obtained on plotting the glucose loss against concentration of acid in the case of phosphoric acid is unlike that obtained from the other inorganic acids, but similar to that obtained from the organic acids.

The results with organic acids are similar to those obtained with the inorganic, but there is a greater glucose loss with each concentration.

The degree of imbibition of liquid by the dry peas is the same as with inorganic acids both quantitatively and qualitatively. These two sets of results again show that glucose removal is not solely a matter of permeability. The most powerful agent in these experiments was acetic acid which at 0·125 *N* removed 0·78 g. glucose from 100 g. peas.

The shape of the curves obtained from these results is unlike that from the inorganic acids, there being no dip after the maximal rise at 0·06 *N* except for a small one in the case of formic acid and a pronounced one with oxalic acid. These two acids give curves similar to that obtained with phosphoric acid. In no case was there anything comparable with the second rise in glucose removal seen with HCl, HNO_3 and H_2SO_4 between 0·25 *N* and 0·5 *N*.

The fall of glucose with the stronger solutions of oxalic acid was unexpected in view of the statement so often put forward in cookery books and by Hutchison [1927] that the presence of calcium causes a decrease in the permeability of peas when soaked. Therefore peas were soaked in an 0·25 *M* solution of $CaCl_2$; this gave 0·14 g. glucose per 100 g. peas and a residual fluid of 137 cc., whilst the control with distilled water gave 0·15 g. glucose in 147 cc. residual fluid. Hence the extra calcium had no effect on loss of glucose, but depressed water imbibition, again showing the independence of the two phenomena.

This result led to further experiments with salts in the soaking liquids.

Alkalis.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
NaOH	140	0·16	140	0·11	140	0·08	136	0·07	136	0·07	134	—	130	—
Na_2CO_3	146	0·16	139	0·14	140	0·17	140	0·16	142	0·17	146	0·15	152	—
$NaHCO_3$	136	0·13	138	0·13	140	0·11	140	0·11	143	0·11	150	0·11	154	0·19

Of the alkalis sodium hydroxide gave a small decrease in glucose removal, sodium bicarbonate a still smaller decrease, and sodium carbonate had no effect. At the highest concentration of sodium hydroxide and carbonate it was not possible to estimate the glucose as the Benedict's solution was decomposed.

Two of the alkalis behaved in the same way as the acids in decreasing the amount of water taken up by the peas. This was not so with sodium hydroxide, probably because at concentrations greater than 0·06 *N* the peas in the cold became pulpy and difficult to strain from the liquid.

Salts.

Concentration of solution	0		0·015 *M*		0·03 *M*		0·06 *M*		0·125 *M*		0·25 *M*		0·5 *M*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
Sodium tartrate	139	0·17	140	0·16	141	0·15	144	0·15	142	0·15	160	0·13	170	0·14
Sodium bitartrate	140	0·14	142	0·21	144	0·22	146	0·32	145	0·36	150	0·47	154	0·53
Na_2SO_4	142	0·14	142	0·14	144	0·14	146	0·14	160	0·14	170	0·11	175	0·09
$NaHSO_4$	142	0·13	148	0·20	150	0·25	152	0·23	154	0·20	158	0·16	160	0·19
Na_3PO_4	136	0·15	136	0·15	138	—	138	—	146	—	153	—	154	—
Na_2HPO_4	136	0·15	138	0·14	140	0·12	142	0·12	144	0·12	146	0·17	—	—
NaH_2PO_4	138	0·17	138	0·17	142	0·17	142	0·16	144	0·17	150	0·17	160	0·18

With the majority of the salts there was no appreciable difference in the amount of glucose removed from 100 g. peas, as compared with that removed by distilled water. The two exceptions were sodium hydrogen tartrate which gave a steady increase with increase of concentration and sodium hydrogen sulphate which gave a result similar to that with sulphuric acid, *viz.* a rise at the lower concentrations tailing off at the higher concentrations.

All the salts behaved like the acids and alkalis in lessening the amount of water taken up by the dried peas. This was specially marked with sodium tartrate and sodium sulphate which when used at a concentration of 0·5 *M* so decreased the imbibition of water that the peas remained wrinkled after 12 hours' soaking, although the salt concentration in the soaking fluid caused them to become as soft as though cooked.

Effect on rate of cooking.

When peas soaked in 0·5 *N* solutions of various organic and inorganic acids were cooked in vigorously boiling water for 45 minutes they became quite soft, but their outer coats remained hard, and therefore they appeared to be less cooked than the control peas which had been soaked in distilled water. This effect was not seen at 0·25 *N* and lower concentrations where the acid-soaked peas behaved exactly as the controls and were well cooked in 40 minutes.

With the alkalis, soaking in 0·25 *N* solutions in the cold rendered the peas as soft as though cooked and they disintegrated to a pulp on being placed in boiling water. Lower concentrations did not affect the time required for cooking. Similar softening in the cold accompanied by disintegration on heating occurred with sodium tartrate and bitartrate, sodium sulphate and bisulphate and the three sodium phosphates at high concentrations. This softening is therefore not due solely to increased alkalinity since some high salt concentrations, even of acid salts, have the same effect.

Peas soaked in 0·25 *M* $CaCl_2$, NaCl, KCl and distilled water gave identical results as to time of cooking and also in glucose removed during 12 hours' soaking.

From these results it appears that so far as carbohydrate loss is concerned, it is immaterial whether peas are soaked in hard or soft water. Further

the addition of the customary "pinch" of bicarbonate does not alter this slight loss or accelerate the cooking of the soaked peas. Its only effect is the usual attainment of a bright green colour by the peas soaked in a weak solution of alkali.

Although the carbohydrate loss incurred on soaking peas is negligible from the point of food value, it is markedly altered by the presence of acids in the steeping fluid. This alteration is not a direct function of p_H since organic acids of the same normality differ markedly amongst themselves in power to extract glucose from peas, and are 2–4 times more powerful in this respect than inorganic acids of the same normality. Any increase of ionic concentration reduces the water imbibed by dried peas.

Hence the increase in glucose loss with acids is not the result of a simple change in permeability of the cell wall, but is probably due to the liberation of glucose from some preformed combination in the plant cells.

Summary.

1. Dried peas on soaking in distilled water for 18–24 hours lose 0·1–0·15 % glucose, *i.e.* about 0·2 % of their total carbohydrate.

2. This loss is unaltered by the addition of alkalis to the soaking fluid.

3. The addition of acids causes a marked rise in loss of glucose.

4. This glucose loss is more marked with organic acids than with inorganic acids.

5. The uptake of water by dried peas is depressed by the addition of acids, alkalis or salts to the soaking fluid.

6. The rate of cooking of peas is unaltered by previously soaking them in acids, alkalis, or salts unless the concentration of alkali or salt is high enough to soften the whole pea in the cold (this would render the pea inedible).

The expenses of these experiments were defrayed by a grant from the Medical Research Council.

References.

Hutchison (1927). Food and principles of dietetics, p. 232. (Arnold, London.)
Masters (1918). *Biochem. J.* **12**, 231.

CCXVII. THE ENZYMIC SYNTHESIS OF PROTEINS WITH SPECIAL REFERENCE TO THE ACTION OF PEPSIN.

By DAVID PATON CUTHBERTSON
and SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received October 22nd, 1931.*)

The first enzymic synthesis of protein from substances of lower molecular weight was described by Danilewski, who observed [1886] that, when an extract of stomach was added to a concentrated solution of the products of a peptic digest of protein, a precipitate formed. Okunew [1895] obtained similar results. The phenomenon was further investigated by Sawjalow [1901] who gave the name plastein to the precipitate he obtained. Taylor [1907, 1909] digested protamine sulphate with a glycerol extract of the liver of the Pacific coast clam and, after concentration of the digestion products and further addition of the enzyme, obtained at the end of 5 months' incubation a substance which was identified as protamine sulphate.

Regarding the nature of plastein little was known by the earlier workers. Okunew [1895] stated that his digests contained 2 % more water after plastein formation had taken place. Sawjalow [1901] believed that plastein was a more complex substance than its precursors owing to its greater ease of precipitation by metallic salts. Henriques and Gjaldbäk [1911, 1912] found that there was always a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. They determined the ratio of free amino-nitrogen to total nitrogen of purified specimens of plasteins and found the figures to be comparable with those of the native proteins. Wasteneys and Borsook [1925, 2] do not however consider this ratio to be a fair criterion of the complexity of protein substances.

Morrell, Borsook and Wasteneys [1927] demonstrated that the rate of hydrolysis of a protein by pepsin, as measured by the amount hydrolysed in the first half hour, diminishes as the concentration of protein increases. Wasteneys and Borsook [1925, 2] confined their attention to the action of pepsin upon concentrated peptic digests of egg-albumin. They found that plastein formation did not occur if the concentration of the digests were less than 2 g. of nitrogen per 100 cc. These workers [1925, 4] were also the first accurately to determine the optimum p_H for plastein formation. They ob-

tained the greatest yields of plastein from peptic digests of egg-albumin at p_H 4·0 and found that the yield decreased very rapidly on either side of this value.

The present writers have further examined this problem and have extended the investigations to other proteins.

Analytical methods.

The peptic digests were prepared as follows.

To 1000 cc. of water, 25 g. of protein were added followed by sufficient hydrochloric acid to bring the p_H to 1·7. This mixture was incubated for about 6 days at 37° with 0·5 % pepsin. After incubation the whole was placed in a boiling water-bath for 30 minutes to inactivate the enzyme and when cool, filtered. The filtrate was concentrated *in vacuo* at 40° until it had a concentration of 3–4 g. nitrogen per 100 cc.

The amount of plastein formation was determined by the method of Wasteneys and Borsook [1925, 1]. 5 cc. of the digest were diluted to 25 cc. and nitrogen was determined in a 5 cc. sample. To another 5 cc. sample of the digest 15 cc. water were added, then 2·5 cc. of 20 % trichloroacetic acid and finally the whole was diluted to 25 cc. and mixed. The plastein was filtered off and the nitrogen content of 5 cc. of the filtrate was determined. From these two results the percentage plastein formation was calculated.

Influence of hydrogen ion concentration.

Specimens of concentrated peptic digests of egg-albumin, serum-albumin and caseinogen were divided into a series of 10 cc. portions and the p_H of each roughly adjusted by the addition of either dilute HCl or NaOH so that a range from about p_H 2·0 to 6·5 was obtained. The specimens were adjusted to the same volume by the addition of the requisite amount of water. 1 g. of pepsin and a few drops of toluene were added to each. They were stoppered and incubated at 37° for 3 days, being shaken at intervals to facilitate solution of the pepsin. The amount of plastein formation was then determined. The p_H of each specimen was determined electrometrically after the pepsin had dissolved.

The optimum p_H for plastein formation in all peptic digests of the three proteins examined was slightly over 4·0. This is in agreement with the results obtained by Wasteneys and Borsook [1925, 4] for peptic digests of egg-albumin.

The influence of temperature.

Sawjalow [1901] and Henriques and Gjaldbäk [1912] had observed from qualitative experiments that an increase in temperature led to an increase in plastein formation. Robertson [1909] and Wasteneys and Borsook [1925, 3] demonstrated this quantitatively. Plastein formation does not increase indefinitely with temperature, since pepsin is inactivated above 72°. Wasteneys

and Borsook [1925, 3], controlling the p_H and using concentrated peptic digests of egg-albumin, obtained their maximum yield of plastein at 72°, no plastein being formed at 80°.

This phenomenon has been re-investigated, using concentrated peptic digests of egg-albumin and serum-albumin. The digests were adjusted to p_H 4·0 and, before addition of the enzyme, were allowed to come to the temperature at which the reaction was to be studied. Plastein formation was determined after 24 hours' incubation. The results with both proteins were in entire agreement with those of Wasteneys and Borsook [1925, 3].

The effect of enzyme concentration.

The concentration of the enzyme has a marked influence upon the amount of plastein formation. The data obtained are shown in Table I for egg-albumin and serum-albumin.

Table I.

	Concentration of enzyme (g. per 100 cc.)	% plastein formation
Concentrated peptic digest of egg-albumin	0·1	7·9
Total nitrogen—3·94 g. per 100 cc.	0·5	13·2
p_H—4·0	1·0	17·8
Incubated 3 days at 37°	2·0	20·3
	5·0	24·5
	10·0	27·4
Concentrated peptic digest of serum-albumin	0·1	7·9
Total nitrogen—3·75 g. per 100 cc.	0·2	12·8
p_H—4·0	0·5	13·9
Incubated 3 days at 37°	1·0	18·7
	5·0	21·9
	10·0	24·8

Ratio of decrease of amino- and carboxyl-groups.

So far the only criterion of plastein formation which has been considered, is the property ascribed exclusively to proteins of insolubility in 2 % trichloroacetic acid. Other confirmatory evidence therefore seemed essential.

Henriques and Gjaldbäk [1912] observed a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. Oda [1926] contested the view that there was any decrease in the free amino-nitrogen. A decrease in the free amino-nitrogen alone cannot however be taken as evidence of the formation of a more complex molecule. It must be shown that there is no simultaneous formation of ammonia such as could be explained by a hydrolysis of amino-groups. Wasteneys and Borsook [1925, 5] found that if either peptic digests of egg-albumin, glycine solutions or solutions of glycine and phosphate were incubated at 37° with high concentrations of glucose, a marked decrease in free amino-nitrogen was observed when the mixtures were more alkaline than p_H 7·0, the effect increasing with increasing alkalinity. They were unable to account for this disappearance of free amino-nitrogen as ammonia, urea or cyanic acid. In the case of the peptic digests, this

diminution in the free amino-nitrogen did not involve any formation of more complex products of protein nature. It must be noted that none of these changes was obtained in the region of p_H 4, the optimum for plastein formation by pepsin.

Steudel, Ellinghaus and Gottschalk [1926] and Steudel and Ellinghaus [1927] state that carboxyl groups are liberated five times as fast as amino-groups during hydrolysis of a protein by pepsin. Felix and Harteneck [1927], however, using histone and Weber and Gesenius [1927] using caseinogen have found that during peptic hydrolysis of these proteins, amino- and carboxyl groups are liberated at equal rates. This was confirmed by Waldschmidt-Leitz and Künster [1927] for egg-albumin, caseinogen, gelatin and histone, and by Sørensen and Katschioni-Walther [1928] for caseinogen, gelatin and gliadin. Cannan and Muntwyler [1930] found that the acid- and alkali-binding powers of gelatin increased at equal rates during peptic digestion.

It is to be expected that during synthesis of a protein, a reversal of the above process will take place, namely, that the concentration of the amino- and carboxyl groups will decrease at the same rate. Rona and Chrometzka [1927] found that although there was a reduction in the free amino-nitrogen during plastein formation there were only indefinite variations in the concentrations of the carboxyl groups. They sought to explain the phenomenon of plastein formation as a deamination, caused by the liberation of amino-groups to form ammonia, which they determined in the digests by aeration with sodium hydroxide or baryta at 70°. Such drastic treatment would however presumably lead to ammonia formation so that the results obtained cannot be relied upon. Rona and Oelkers [1928] indeed re-investigated the problem and found that there was an equivalent decrease of amino- and carboxyl groups during plastein formation, indicating that the latter is actually a synthesis.

Experiments were made by the following procedure to elucidate this point.

The reaction of the digest to be examined was adjusted so that upon addition of pepsin to a concentration of 10 % the mixture had p_H 4. 0·5 g. of finely divided pepsin was weighed into each of two 25 cc. volumetric flasks, followed by 5 cc. of digest. The contents of one flask were diluted immediately to 25 cc. and well shaken until all was dissolved, and the amino-nitrogen, carboxyl groups and ammonia were determined; these values were taken as the initial values before plastein formation. A drop of toluene was added to the contents of the second flask, the flask stoppered and incubated at 37°. After 2 days the contents of this flask were diluted to 25 cc., well mixed and the amino-nitrogen, carboxyl groups and ammonia again determined. The nitrogen insoluble in 2 % trichloroacetic acid was determined upon a sample from the second flask.

Amino-nitrogen was determined by the method of Linderström-Lang [1928]. The error due to separation of peptones *etc.* in presence of acetone being overcome by carrying out a second titration in which sufficient 0·1 *N* HCl was added prior to the addition of acetone, so that only 1–2 drops of

0·1 *N* acid were necessary to complete the titration. Using this technique consistent results could always be obtained. A blank determination using 1 cc. of distilled water was always carried out, and this correction was allowed for. For carboxyl groups the method employed was that of Willstätter and Waldschmidt-Leitz [1921].

Ammonia was determined as follows: in an 8 × 1 inch pyrex test-tube 1–5 cc. of the solution to be examined were placed. The contents of the tube were diluted if necessary to about 5 cc. with distilled water. 2 cc. of saturated borax solution were added followed by a few drops of liquid paraffin and a glass bead to prevent bumping. A spiral of galvanised iron wire was placed in the tube. A delivery tube was fitted, the end of which dipped under 2 cc. of 0·05 *N* HCl, contained in a test-tube graduated at 25 cc. The ammonia was distilled over by boiling for 4 minutes over a micro-burner. Frothing was prevented by the use of the liquid paraffin and spiral of iron wire. After the distillation, the delivery tube was disconnected and washed into the receiving tube, the contents of which were diluted to about 20 cc. After cooling, the ammonia was determined colorimetrically by Nessler's reagent.

In distilling peptic digests with borax, ammonia from ammonium salts only is liberated, the borax being too weak an alkali to attack the amino-groups. This was proved experimentally with many amino-acids.

The results of these investigations are shown in Table II. The concentra-

Table II.

Total nitrogen (g. per 100 cc.)	Plastein formation %	Amino-nitrogen (cc. *N* HCl %)	Carboxyl (cc. *N* KOH %)	Decrease in NH_2 and COOH: NH_2 (cc. *N* HCl %)	Decrease in NH_2 and COOH: COOH (cc. *N* KOH %)	Ratio of decrease of $\frac{COOH}{NH_2}$	Concentration of ammonia (mg. N per 100 cc.)	Hours
A. Egg-albumin digest. Incubation 2 days at 37°, p_H 4:								
5·139	—	81·0	115·5	—	—	—	36·3	0
5·157	20·7	74·5	109·5	6·5	6·0	0·93	37·1	48
4·419	—	73·0	107·5	—	—	—	48·3	0
4·421	17·2	65·0	98·7	8·0	8·8	1·1	48·7	48
4·160	—	72·0	80·5	—	—	—	25·4	0
4·089	10·5	61·5	69·0	10·5	11·5	1·09	27·1	48
5·198	—	94·3	128·5	—	—	—	30·3	0
5·211	19·7	80·9	115·5	13·4	13·0	0·97	32·6	48
B. Serum-albumin digest. Incubated 2 days at 37°, p_H 4:								
3·516	—	70·2	120·9	—	—	—	56·3	0
3·522	16·4	64·3	115·4	5·9	5·5	0·94	58·1	48
4·144	—	84·3	158·5	—	—	—	49·7	0
4·098	21·4	74·7	148·7	9·6	9·8	1·02	50·3	48
3·917	—	78·0	144·2	—	—	—	61·3	0
4·012	18·7	66·6	133·3	11·4	10·9	0·96	63·1	48
2·961	—	63·4	110·0	—	—	—	27·4	0
3·012	12·8	55·1	102·5	8·3	7·5	0·90	27·8	48
C. Caseinogen digest. Incubated 2 days 37°, at p_H 4:								
3·722	—	87·5	111·5	—	—	—	42·4	0
3·810	10·2	82·1	106·9	5·4	5·6	1·04	43·1	48
3·019	—	80·3	102·4	—	—	—	34·6	0
2·998	8·6	76·1	97·9	4·2	4·5	1·05	34·8	48
4·526	—	90·7	124·6	—	—	—	45·7	0
4·492	11·4	82·4	116·7	8·3	7·9	0·95	46·0	48
4·259	—	86·6	105·4	—	—	—	41·6	0
4·271	9·6	80·1	99·5	6·5	5·9	0·93	41·2	48

tions of the amino- and carboxyl groups are expressed in terms of cc. *N* acid or alkali respectively per 100 cc. digest. The two flasks containing digest and pepsin used for each experiment always contained slightly different amounts of nitrogen, but the amino-nitrogen, carboxyl and ammonia figures of the first flask have been corrected to correspond to the nitrogen figure of the second flask. The corrections, however, were very small, being almost negligible.

It will be seen from the results that in all cases plastein formation is accompanied by an equivalent decrease in the concentration of amino- and carboxyl groups. It appears therefore that plastein formation involves a peptide linking of these groups. These results further strengthen the view that plastein formation is essentially a synthesis. No appreciable formation of ammonia takes place during plastein formation. The amount of plastein formation as determined by the quantity of nitrogen insoluble in 2 % trichloroacetic acid does not appear, however, to run parallel with the decrease in concentration of the amino- and carboxyl groups. This may be due to the fact that in measuring the former, only one phase of the reaction is being determined. Wasteneys and Borsook [1925, 2] have shown that during plastein formation all the fractions of a protein hydrolysate appear to be involved, there being an increase of certain fractions such as proteoses and peptones from molecules of less complexity.

The effect of the degree of enzymic hydrolysis on the process of re-synthesis.

Sawjalow [1901] concluded that the nearer a peptic digest of protein approaches in complexity to the original protein, the more easily does plastein formation take place. Henriques and Gjaldbäk [1912] and Wasteneys and Borsook [1930] controlling the p_H reached similar conclusions. We have re-investigated this point, using serum-albumin.

A peptic digest of serum-albumin was prepared and at intervals of 5, 10, 20 and finally 30 days portions were removed, the active enzyme destroyed by heat, the unchanged protein filtered off and the filtrate concentrated *in vacuo*. The samples were concentrated to approximately the same degree. Pepsin was added to the concentrated digests to 10 % concentration and the mixtures brought to p_H 4. The amount of plastein formation was determined at intervals. The results were in agreement with the observations of the above workers.

The character of the original protein and its relationship to plastein formation.

The amount of plastein obtained under comparable conditions varies considerably according to the protein from which the digest has been prepared. While peptic digests of egg- and serum-albumins gave the highest yields, peptic digests of caseinogen were found to give much lower yields. Wasteneys and Borsook [1930] found that peptic digests of gliadin gave much smaller yields than peptic digests of egg-albumin. So far no investigator has obtained

plasteins from peptic digests of gelatin. The present writers have similarly failed. Wasteneys and Borsook [1930] have suggested that this failure may be due to the use of acid in preparing gelatin from collagen since Henriques and Gjaldbäk [1911, 1912] found that partial acid or alkaline hydrolysates of proteins yielded no plasteins. With this in view the present writers attempted to form plasteins from a concentrated peptic digest of collagen.

Tendons were first digested with trypsin to hydrolyse proteins other than collagen. The collagen was then washed with water to remove the disintegration products of the foreign proteins. The collagen was digested with pepsin at 37° and p_H 1·7 for several days. The procedure for preparing the concentrated peptic digest of collagen was essentially the same as described earlier in this paper. The concentrated digest of collagen was finally incubated 7 days at p_H 4 with 10 % pepsin, but no evidence of plastein formation was obtained. The results of this experiment do not appear to support the theory advanced by Wasteneys and Borsook to account for their failure to produce plastein from concentrated digests of gelatin.

Properties and composition of the plasteins.

The plasteins prepared from the various peptic digests all gave positive protein biuret tests. They were soluble in strong acids and strong alkalis and were coagulated by boiling in alkaline solution, but not in acid solution. The plasteins were all digested rapidly by pepsin at p_H 1·7 and from their peptic digests, primary proteoses could be salted out by half saturation with ammonium sulphate. The sulphur, nitrogen and phosphorus contents of plasteins from various sources were determined and compared with those of the original native proteins. It will be seen from the data (Table III) that the contents

Table III.

Source from which plastein was prepared	Sample	Composition of plastein S %	N %	P %	Composition of protein from which digest was prepared S %	N %	P %
Egg-albumin	1	1·63	13·41	—	1·62	15·51	—
	2	1·70	13·82	—	—	—	—
	3	1·58	13·56	—	—	—	—
	Average	1·63	13·59				
Serum-albumin	1	1·91	14·01	—	1·93	15·93	—
	2	1·83	13·89	—	—	—	—
	3	2·10	13·76	—	—	—	—
	Average	1·94	13·89				
Caseinogen	1	0·81	14·45	0·11	0·78	15·6	0·81
	2	0·83	14·02	0·16	—	—	—
	3	0·76	14·76	0·21	—	—	—
	Average	0·80	14·41	0·16			

of these elements in the plasteins and the original proteins are of the same order, with the exception of phosphorus which is almost absent from plasteins derived from caseinogen.

Sulphur was determined by Denis's modification of Benedict's method and phosphorus by the direct weighing of the ammonium phosphomolybdate formed by Neumann's reaction.

In Table IV are shown the cystine content and strength of the Molisch α-naphthol tests both in the plasteins and in the proteins from the digests of which they were derived. The method for the determination of cystine was a modification of the method of Folin and Marenzi [1929] and is described in a subsequent note by one of us [Tompsett, 1931].

Table IV.

	Cystine %	Molisch test
Egg-albumin	2·29	+ +
Plastein (egg-albumin digest)	3·07	+ +
Serum-albumin	2·34	+ +
Plastein (serum-albumin digest)	3·84	+ +
Caseinogen	0·38	faintly +
Plastein (caseinogen digest)	1·35	+ +
Pepsin	0·78	+ +

Discussion.

It has been shown that when peptic digests of egg-albumin, serum-albumin and caseinogen are concentrated to about 3–4 g. nitrogen per 100 cc., upon addition of pepsin and incubation at 37°, an apparent synthesis of protein results, the maximum synthesis being obtained at p_H 4.

The amount of protein or plastein as it has been termed is also influenced by the temperature and the concentration of the enzyme.

Plastein is insoluble in 2 % trichloroacetic acid, gives a positive biuret test and its formation is accompanied by an equivalent decrease in the concentrations of amino- and carboxyl groups without any formation of ammonia. It appears then that plastein formation is actually a synthesis of protein from protein decomposition products. The highest yields were obtained from egg-albumin and serum-albumin digests, caseinogen digests giving much smaller yields, while gelatin digests produced no plastein at all.

The facts that Henriques and Gjaldbäk [1911, 1912] have been unable to obtain plasteins by the action of pepsin upon partial acid or alkaline digests of proteins and that these investigators and also Wasteneys and Borsook were unable to prepare plasteins by the action of trypsin upon tryptic digests of proteins are of great interest. It is curious too that the longer pepsin acts upon a protein during the hydrolytic stage, the lower is the yield of plastein from the subsequently concentrated digest. The possibility arises that pepsin can only unite those products of protein decomposition containing certain groupings in their structure. While little information was derived from the determination of the percentage of sulphur, nitrogen and phosphorus in the plasteins, the figures obtained being of the order of those of the native proteins from which the plasteins were derived, more information was derived from

the analyses of the plasteins for cystine and also by the strength of the Molisch test for carbohydrate. In each case the plasteins contained a higher percentage of cystine than the corresponding native protein. Serum- and egg-albumin both gave strong Molisch tests as did their corresponding plasteins, but the plasteins from caseinogen digests all exhibited stronger tests than the original caseinogen which gave only a faint reaction. Doubt has often been expressed as to whether the protein molecule really contains a carbohydrate or not. Recently, however, Rimington [1929, 1931] and Levene and Mori [1929] have isolated carbohydrate complexes from purified proteins which appear to consist of glucosamine and mannose units and are present to the extent of about 2 %. The question arises as to the importance of cystine and the carbohydrate group of the protein molecule in the formation of plastein. It is suggestive that serum- and egg-albumin contain fairly high percentages of cystine and give strong Molisch tests, while caseinogen contains a very low percentage of cystine and gives a faint Molisch test and gelatin gives no Molisch test and contains no cystine.

Summary.

1. The optimum hydrogen ion concentration for the synthesis of plastein from concentrated digests of egg- and serum-albumin and also caseinogen, incubated at 37°, was p_H 4.

2. The amount of plastein formed from egg- and serum-albumin digests increased rapidly with temperature, reaching a maximum at 70°.

3. Increase in the concentration of enzyme increased plastein formation in concentrated peptic digests of egg- and serum-albumin.

4. The ratio of the decrease in the amino- and carboxyl groups during the process of plastein formation from egg- and serum-albumin and also caseinogen digests was 1 : 1.

5. There was no appreciable liberation of ammonia during formation of plastein.

6. The longer a protein was allowed to digest the less was the yield of plastein.

7. Peptic digests of egg- and serum-albumin gave better yields than those of caseinogen. Gelatin yielded no plastein from the products of digestion.

8. Certain properties of the plasteins are described.

We are much indebted to the Carnegie Trust for a grant in aid of this research.

REFERENCES.

Cannan and Muntwyler (1930). *Biochem. J.* **24**, 1012.
Danilewski (1886) quoted by Henriques and Gjaldbäk (1911).
Felix and Harteneck (1927). *Z. physiol. Chem.* **165**, 103.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Henriques and Gjaldbäk (1911). *Z. physiol. Chem.* **71**, 485.
—— —— (1912). *Z. physiol. Chem.* **81**, 439.
Levene and Mori (1929). *J. Biol. Chem.* **84**, 49.
Linderström-Lang (1928). *Z. physiol. Chem.* **174**, 275.
Morrell, Borsook and Wasteneys (1927). *J. Gen. Physiol.* **8**, 601.
Oda (1926). *J. Biochem. (Japan)*, **6**, 77.
Okunew (1895) quoted by Henriques and Gjaldbäk (1911).
Rimington (1929). *Biochem. J.* **23**, 430.
—— (1931). *Biochem. J.* **25**, 1062.
Robertson (1909). *J. Biol. Chem.* **5**, 393.
Rona and Chrometzka (1927). *Biochem. Z.* **189**, 249.
—— and Oelkers (1928). *Biochem. Z.* **203**, 298.
Sawjalow (1901) quoted by Wasteneys and Borsook (1930).
Sørensen and Katschioni-Walther (1928). *Z. physiol. Chem.* **174**, 251.
Steudel and Ellinghaus (1927). *Z. physiol. Chem.* **166**, 84.
—— —— and Gottschalk (1926). *Z. physiol. Chem.* **154**, 21, 198.
Taylor (1907). *J. Biol. Chem.* **3**, 87.
—— (1909). *J. Biol. Chem.* **5**, 381.
Tompsett (1931). *Biochem. J.* **25**, 2014.
Waldschmidt-Leitz and Künster (1927). *Z. physiol. Chem.* **171**, 70, 290.
Wasteneys and Borsook (1925, 1). *J. Biol. Chem.* **62**, 1.
—— —— (1925, 2). *J. Biol. Chem.* **62**, 15.
—— —— (1925, 3). *J. Biol. Chem.* **62**, 633.
—— —— (1925, 4). *J. Biol. Chem.* **62**, 675.
—— —— (1925, 5). *Biochem. J.* **19**, 1128.
—— —— (1930). *Physiol. Rev.* **10**, 110.
Weber and Gesenius (1927). *Biochem. Z.* **187**, 410, 429.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54** B, 2988.

CCXVIII. A NOTE ON THE DETERMINATION OF CYSTINE IN PROTEINS BY THE METHOD OF FOLIN AND MARENZI.

By SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received October 22nd, 1931.*)

There have been many modifications of the original method of Folin and Looney [1922] for the determination of cystine in proteins. Folin and Trimble [1924] made the method more specific for cystine by removing most of the molybdenum compounds from the phosphotungstic acid reagent thereby decreasing its sensitivity towards tryptophan, tyrosine and phenols. Folin and Marenzi [1929, 1] have recently stated that it is necessary to add much more of the reagent than employed by Folin and Looney and also recommend adding the sulphite before the alkali since a smaller amount is required. Folin and Marenzi [1929, 2] have published the details for the preparation of the phosphotungstic reagent free from all traces of molybdenum. Rimington [1930] examining this method found that a turbidity often formed in the standard and the test solutions, and that the intensity of the colour varied appreciably with slight changes in the concentration of sodium carbonate. Folin [1930] recommended the use of urea for the prevention of this turbidity. Rimington [1930] found this to be quite satisfactory for concentrations of sodium carbonate up to 3*N*. Hunter and Eagles [1927] have recommended the use of sodium hydroxide in place of sodium carbonate.

The present writer found that even when urea was used as recommended by Folin [1930] turbidities often occurred in the test and standard solutions in the Folin-Marenzi method. It was found however that a blue colour developed when sodium bicarbonate solutions were used in place of sodium carbonate. The colour obtained was stronger than that with sodium carbonate and turbidity did not occur even after 30 minutes. It was therefore decided to test the possibility of replacing the carbonate by bicarbonate.

The following solutions were used:

(1) phosphotungstic reagent of Folin and Marenzi [1929, 2];
(2) 20 % sodium sulphite;
(3) saturated sodium bicarbonate solution;
(4) standard cystine solution, containing 1 mg. in 1 cc. *N* H_2SO_4.

Into each of three 100 cc. volumetric flasks were measured 2 cc. of standard cystine solution followed by 2 cc. of sulphite solution. After allowing to stand 1 minute to ensure reduction of cystine to cysteine, bicarbonate solution was added as follows: to the first flask 5 cc., to the second 10 cc., and to the third 20 cc. Then 8 cc. of phosphotungstic reagent were added to each, and after 8 minutes they were diluted to 100 cc. and compared in a colorimeter. These three solutions were identical. Similar results were obtained using 1 cc. and 4 cc. of standard respectively, the colour being proportional to the concentration of the cystine in the range tested, *i.e.* 1–4 mg. Turbidities were never encountered so that it was unnecessary to use lithium sulphate or urea. The colours obtained remained stable for at least 30 minutes.

A blank determination was carried out and found to be inappreciable. Solutions of tyrosine, tryptophan, glycine, alanine, glutamic acid, aspartic acid and histidine in concentrations greater than those present in protein hydrolysates were tested by this method. No colour developed apart from the slight colour which develops during a blank determination. No colour was developed by uric acid.

From the results shown in Table I it will be seen that there was no appreciable loss when known amounts of cystine were boiled with 20 cc. of 6*N* H_2SO_4 for 18 hours under the same conditions as used in the hydrolysis of the proteins.

Table I.

Exp.	Initial amount of cystine mg.	Amount of cystine determined after hydrolysis mg.
1	10	10·4
2	20	19·3
3	40	39·4

For the determination of cystine in proteins, 1–5 g. of protein were boiled with 20 cc. of 6*N* H_2SO_4 in a 300 cc. Kjeldahl flask under a reflux condenser for 18 hours. After cooling, the hydrolysate was filtered, washed into a 100 cc. volumetric flask and made up to 100 cc. A volume of 5–10 cc. containing about 1–4 mg. cystine was measured into a 100 cc. volumetric flask, followed by 2 cc. of sulphite solution. After 1 minute 20 cc. of saturated sodium bicarbonate solution were added and then 8 cc. of phosphotungstic reagent. After 8 minutes the mixture was diluted to 100 cc. and, after mixing, compared with a suitable standard prepared in the same way.

The cystine contents of a number of proteins were determined and the results are shown in Table II.

Table II.

Protein	Cystine (%)
Gelatin (B.D.H.)	Trace
Caseinogen (B.D.H.)	0·38, 0·41, 0·34 (average 0·38)
Edestin (B.D.H.)	1·46, 1·42, 1·48 (average 1·45)
Egg-albumin (Kahlbaum)	2·29, 2·29, 2·35 (average 2·31)
Serum-albumin (Kahlbaum)	2·29, 2·26, 2·34 (average 2·30)

SUMMARY.

A modification of the Folin-Marenzi method for the determination of cystine in proteins is described in which a solution of sodium bicarbonate takes the place of the carbonate used in the original method.

The colour produced in the presence of bicarbonate is uninfluenced by the concentration of this substance and turbidity has never been experienced.

The cystine contents of certain proteins have been determined.

REFERENCES.

Folin (1930). *J. Biol. Chem.* **86**, 179.
—— and Looney (1922). *J. Biol. Chem.* **51**, 421.
—— and Marenzi (1929, 1). *J. Biol. Chem.* **83**, 103.
—— —— (1929, 2). *J. Biol. Chem.* **83**, 109.
—— and Trimble (1924). *J. Biol. Chem.* **60**, 473.
Hunter and Eagles (1927). *J. Biol. Chem.* **72**, 177.
Rimington (1930). *Biochem. J.* **24**, 1114.

CCXIX. THE HEMICELLULOSES OF THE WOOD OF ENGLISH OAK.

I. THE EFFECT OF THE DRYING OF WOOD ON THE YIELD AND COMPOSITION OF HEMICELLULOSE A.

By MARGARET HELENA O'DWYER.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received October 28th, 1931.*)

Introduction.

In two recent papers Campbell and Booth [1930, 1931] have drawn attention to the chemical changes which take place in wood during various drying treatments, and have shown that these changes are associated principally with the furfuraldehyde-yielding complexes in both the hardwood and softwood examined. Since the hemicelluloses previously investigated [O'Dwyer, 1923, 1926, 1928] consisted largely of such complexes and exhibited marked instability towards heat, it has been decided to examine the general chemical character of the hemicelluloses of the wood of English oak with particular reference in the first instance to the effect on these substances of various methods of drying the original wood. The material used was cut from English oak wood of approximately 40 years' growth from the same locality as that used by Campbell and Booth [1930]. Hemicellulose A was obtained from samples of the wood (*a*) in the green state, (*b*) after air-drying, (*c*) after kiln-drying, and (*d*) after drying to constant weight in an oven at 100°.

Experimental.

I. *Preparation of material.*

(*a*) *From green wood.* A portion of each of the sapwood and heartwood from the original wood was converted to sawdust as soon as possible after felling, and the preparation of hemicelluloses commenced without delay. After conversion, the sapwood attained a moisture content of 25·0 % and the heartwood 27·5 %.

(*b*) *From air-dried wood.* A portion of the original wood was converted to 1-inch boards, piled, and air-dried for several months. Samples of sawdust were prepared from sapwood and heartwood as before. The sawdust was

screened in each case and the 60–80 mesh material was reserved for the preparation of the hemicelluloses. After conversion the moisture content of the sapwood was 12·28 %, and that of the heartwood 12·82 %.

(*c*) *From kiln-dried wood.*

1. *Mild treatment.* A third portion of the original wood was converted to 1-inch boards and kiln-dried according to the following schedule.

i. *Schedule employed in mild kiln-drying treatment.*

Temperature ° C.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
35·0	85	5	5	60–40
35·0	80	2	7	40–35
37·8	75	1	8	35–30
37·8	65	2	10	30–25
40·6	60	4	14	25–15
40·6	60	3	21	10

The rate of initial warming of the boards was 3° per hour at a constant humidity of 85 %. The moisture content after conversion to 60–80 mesh sawdust was, sapwood 10 %, heartwood 11·37 %. A slight darkening of the wood occurred as a result of the seasoning.

2. Several 1-inch boards were subjected to a comparatively drastic kiln-drying treatment according to the following schedule.

ii. *Schedule employed in drastic kiln-drying treatment.*

Temperature ° C.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
46·1	80	2½	2½	60–40
46·1	75	1	3½	40–35
46·1	70	1	4½	35–30
46·1	65	1	5½	30–25
46·1	60	1	6½	25–20
48·9	55	2	8½	20–15
51·7	50	2	10½	15–10
54·4	50	2	12½	10

The rate of initial warming of the boards was 5° per hour at a constant humidity of 80 %. A decided darkening of the wood was observed after this treatment. The moisture content after conversion to 60–80 mesh sawdust was, sapwood 9 %, heartwood 10·9 %.

(*d*) *From oven-dried wood.* Samples of sawdust prepared from the sapwood and heartwood of the original wood were placed on trays and dried to a constant weight at 100°. This required about 24 hours for each batch.

The sawdust was much darker in colour than that obtained from green wood. The moisture contents after preparation of 60–80 mesh material were, sapwood 7·2 %, heartwood 9·3 %.

II. *Preparation of hemicelluloses.*

The method described for the preparation of these substances from beech wood [O'Dwyer, 1926, 1928] was followed in the present instance, amounts of 60–80 mesh wood flour of from 500 to 1000 g. being employed. The addition of lime water to the sodium hydroxide extract was, however, omitted, the first extraction for 4 hours under a mechanical stirrer with 0·2 % sodium hydroxide being followed by a second, lasting 48 hours, which removed the greater part of hemicellulose B. Hemicellulose A, which it has been found possible to separate incompletely into two different fractions, was removed by three subsequent extractions of the wood with 4 % sodium hydroxide for 48 hours under a stirrer at room temperature. Ground-glass filters were used throughout the work except in the case of the sawdust, which was filtered directly on to the Büchner, no cloth or filter-paper being employed.

The amounts of material extracted with hot water and sodium hydroxide respectively were estimated as percentages of the oven-dried weight of the appropriate wood samples, and furfuraldehyde was determined by the phloroglucinol method in each hemicellulose fraction and wood residue. The analytical results are given in Table I.

Table I. *The relationship between the respective pentosan contents of oak wood and of the hemicellulose derived therefrom before and after various drying treatments.*

(Results expressed as percentages of the oven-dried weight of the respective wood samples.)

	Green		Air-dried		Kiln-dried (mild)		Kiln-dried (drastic)		Oven-dried		
	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Remarks
Hot water extract	6·8	10·2	9·1	12·7	10·6	11·0	11·2	9·0	7·0	9·1	—
Total NaOH extract	15·6	14·8	13·4	12·6	16·8	18·6	20·2	25·7	22·7	22·5	—
Yield of hemicellulose A	10·0	7·3	8·5	6·8	7·0	7·8	6·5	5·5	5·8	3·0	—
Total pentosan content of wood before extraction	24·75	24·88	23·76	23·42	23·74	23·11	—	—	23·61	23·78	Data obtained by Campbell and Booth [1930] for oak wood from the same locality
Pentosan content of hemicellulose A	8·73	6·28	6·55	6·34	5·39	6·93	4·43	5·32	4·85	2·82	—
Total pentosan content of wood after extraction	12·9	12·0	13·3	12·9	12·7	12·2	12·8	11·95	12·9	12·4	—

Tests for methyl alcohol.

As the hemicellulose B isolated from wood on previous occasions appeared to bear a strong resemblance to the pectic substances [O'Dwyer, 1926], the various sodium hydroxide extracts from oak were submitted to the test for methyl alcohol described by Schryver and Wood [1920] in order to ascertain whether de-esterification had taken place under conditions in which it would occur in the case of these substances [Branfoot, 1929].

Table II shows the results obtained, the appearance of the pink colour being taken as an indication of the presence of methyl alcohol.

Table II. *Tests for methyl alcohol in sodium hydroxide extracts of oak wood after various drying treatments.*

	Green		Air-dried		Kiln-dried (mild)		Kiln-dried (drastic)		Oven-dried	
	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart
1st extraction 0·2 % NaOH	+ +	+	+	+	+	+	−	+	−	−
2nd extraction 0·2 % NaOH	−	−	−	−	−	−	−	−	−	−
4 % NaOH extraction	−	−	−	−	−	−	+	+	+ +	+ +

\+ indicates that test is positive for methyl alcohol.
\+ + indicates that test is strongly positive for methyl alcohol.
− indicates that test is negative for methyl alcohol.

Discussion of results.

As will be seen from Table I, the method of drying oak wood appears to influence both the yield and the constitution of hemicellulose A. In the first place, although the percentage amount of material extracted from the wood during the preparation of the hemicelluloses increases with increasingly drastic methods of drying, the yield of hemicellulose A actually becomes less. The yield from oven-dried wood is approximately half that obtained from the green material.

Owing to the colloidal nature of the hemicelluloses a considerable amount of loss occurs during their preparation. This is most marked in the case of the green wood, so that the disparity shown in Table I between the yields of hemicellulose A after the various methods of drying is in reality greater than would appear from the figures given. The furfuraldehyde-yielding complexes have been considered as pentosan at this stage of the work, but further study of these complexes will probably show them to consist, to some extent, of uronic acid anhydrides, as in the case of beech wood [O'Dwyer, 1926]. Hemicellulose A from oak heartwood appears to be somewhat less soluble in 4 % NaOH than that from the sapwood, as the latter could be almost completely extracted in one operation. According to Anderson [1931] higher yields of hemicellulose can be obtained from the wood of birch and spruce by extraction with 5 % NaOH in the cold, followed by an extraction with 7 % NaOH on a boiling water-bath, but it is certain that these extracts contain a high proportion of wood components other than hemicelluloses.

After drastic kiln-drying and oven-drying of oak wood, the greater part of the substances other than the hemicelluloses extracted by sodium hydroxide from the wood appeared to combine with the sodium hydroxide to form a salt or salts, not removable by dialysis, as these substances, when dry, contained a high percentage of ash which was largely composed of sodium carbonate. This occurred to a much less extent both in air-dried wood and in that submitted to a mild kiln-drying treatment. It was also noted that under

the more drastic conditions of drying the addition of a much smaller amount of acetic acid was necessary for the neutralisation of the sodium hydroxide extracts and the consequent precipitation of the hemicelluloses. In the case of the green wood, by far the greater part of the acetic acid is recoverable as sodium acetate. It is not improbable that the hemicelluloses combine to some extent with the sodium hydroxide in accordance with the suggestion by Neale [1931] that certain hydroxyl groups of carbohydrates may exhibit weakly acidic properties. This property of these groups has long been recognised by workers on the sugars. It is certain, however, that the addition of acetic acid breaks down any such union since the ash content of hemicellulose A is under 2 % and has been identified as silica with traces of phosphorus.

The presence of methyl alcohol in the sodium hydroxide extracts from which hemicellulose A was obtained would appear to support previous suggestions [O'Dwyer, 1926, 1928] that the hemicelluloses may be transitional products in the transformation of pectin to lignin in the plant. Since methyl alcohol is present in the more concentrated alkali extracts obtained from kiln-dried and oven-dried wood (Table II) it is conceivable that during drying under comparatively severe conditions demethylation is carried further. This is borne out in the work of Campbell and Booth [1930] where it is shown that the methoxyl content of kiln- and oven-dried wood is lower than that of either green or air-dried wood. It will be interesting to note whether the percentage amount of hemicellulose B increases as a result of severe drying conditions, or whether other substances more akin to lignin are formed. Campbell and Booth [1930, 1931] have established an apparent increase in the lignin content of wood during drying, and Klason [1930] suggests that the pentoses, and of these especially xylose, may be regarded as the parent substance of lignin, and further that the pentoses are partly methylated in the leaves of plants.

From the foregoing considerations it is to be concluded that during the drying of timber certain definite chemical changes take place in the hemicelluloses. With increasingly drastic conditions of drying the total alkali-solubility of the wood increases and the yield of hemicellulose A decreases, and thus it is apparent that the hemicellulose A of green wood is transformed into a substance or substances of a different nature as wood dries. The full significance of this transformation may well be far-reaching, and a more detailed investigation of it is in progress.

Summary.

1. Hemicellulose A has been prepared from the sapwood and heartwood of English oak before and after various methods of drying.

2. With increasingly severe conditions of drying, the hot water and sodium hydroxide extracts of wood increase in amount, and the yields of hemicellulose A decrease.

3. It is suggested that under drastic drying conditions hemicellulose A is altered in constitution and gives rise to decomposition products which have not been identified.

The author wishes to express her indebtedness to Mr R. A. G. Knight of the Seasoning Section, Forest Products Research Laboratory, for carrying out the drying treatment, to the late Prof. Perkin and to Prof. Robinson for facilities afforded in the Dyson Perrins Laboratory, Oxford, to Mr W. G. Campbell of the Chemistry Section, Forest Products Research Laboratory, for many helpful suggestions, to Sir James Irvine for criticising the manuscript, and to Mr R. S. Pearson, Director of Forest Products Research for permission to publish these results.

REFERENCES.

Anderson (1931). *J. Biol. Chem.* **41**, 559.
Branfoot (1929). *Food Investigation Special Report No.* 33 (*D.S.I.R.*), pp. 36–39.
Campbell and Booth (1930). *Biochem. J.* **24**, 641.
—— —— (1931). *Biochem. J.* **25**, 756.
Klason (1930). *Ber. deutsch. chem. Ges.* **63 B**, 1548.
Neale (1931). *J. Soc. Chem. Ind.* **50**, 117 T.
O'Dwyer (1923). *Biochem. J.* **17**, 501.
—— (1926). *Biochem. J.* **19**, 656.
—— (1928). *Biochem. J.* **22**, 381.
Schryver and Wood (1920). *Analyst*, **45**, 164.

CCXX. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

II. THE EFFECT ON WOOD SUBSTANCE OF *ARMILLARIA MELLEA* (VAHL.) FR., *POLYPORUS HISPIDUS* (BULL.) FR., AND *STEREUM HIRSUTUM* FR.

By WILLIAM GEORGE CAMPBELL.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received October 28th, 1931.*)

In a previous communication [Campbell, 1930] it has been pointed out that comparatively little experimental work has been done on the chemistry of the particular type of wood decay in which lignin is decomposed, and further, that it is unknown whether such decomposition is invariably accompanied by simultaneous decomposition of the wood carbohydrates. Analytical data were obtained, however, which showed clearly that in the decay of wood by *Polystictus versicolor* (Linn.) Fr. carbohydrates are depleted as well as lignin. Falck [1927] has concluded that in wood decayed by *Fomes annosus* cellulose is decomposed in the advanced stages. In the present investigation the study of the so-called white rots has been continued by examining the respective effects of *Armillaria mellea* (Vahl.) Fr. on beech wood, *Polyporus hispidus* (Bull.) Fr. on ash heartwood, and *Stereum hirsutum* Fr. on oak sapwood.

Experimental.

(1) The experimental procedure and analytical methods were the same as those employed in the previous work. Each sample of 60–80 mesh wood flour of known composition was first sterilised, then inoculated with the appropriate fungus and incubated at 20°. After decay had proceeded to the required stage, the wood residues were washed with cold water until free from acid, dried and sterilised at 105°. The comparative effects of 1 % sodium hydroxide on decayed and sound wood respectively were examined only in the case of beech wood. All analytical results are expressed as percentages by weight of the oven-dry original wood, except where it is stated otherwise.

(2) It was noted that oak sapwood decayed by *Stereum hirsutum* was much lighter in colour than the original sound wood, and furthermore, that in various localities the decayed residue was almost pure white in colour. As a rule these white patches were quite small, but in one culture flask there occurred an unusually large patch of white residue.

Table I. *The effect of 1 % NaOH on beech wood before and after decay by* Armillaria mellea.

	Original wood	Decayed wood	Original wood after alkali-extraction	Decayed wood after alkali-extraction
Duration of decay	—	16 months	—	—
Loss due to decay *plus* cold water washing	—	8·66	—	—
Cold water-soluble	0·64	0·17	—	—
Hot water-soluble	1·65	0·89	—	—
1 % NaOH-soluble	16·63	15·88	—	—
Cellulose	57·49	50·84	58·67	50·10
Lignin	23·79	24·00	19·79	18·42
Pentosans in cellulose	15·50	12·62	18·42	14·65
Pentosans not in cellulose	11·45	11·22	0·60	4·98
Methoxyl content	6·33	5·29	4·81	4·27

By carefully cutting the flask it was possible to remove the greater part of this white material. It was found to have retained the cellular structure of the original wood flour, and when dried to constant weight *in vacuo* over $CaCl_2$ and P_2O_5 it weighed 0·1668 g. After extraction with alcohol-benzene, a standard lignin determination was applied, using the appropriate volume of 72 % H_2SO_4. The lignin content of the sample was 0·0212 or 12·5 %. For

Table II. *Analysis of 60–80 mesh oak sapwood and ash heartwood before and after decay by* Stereum hirsutum *and* Polyporus hispidus *respectively.*

	S. hirsutum		*P. hispidus*	
	Original wood	Decayed wood	Original wood	Decayed wood
Duration of decay	—	13 months	—	12 months
Loss due to decay *plus* cold water washing	—	20·16	—	11·3
Cold water-soluble	2·97	1·40	1·38	0·90
Hot water-soluble	5·02	3·77	3·02	2·32
1 % NaOH-soluble	18·66	18·64	21·13	19·45
Cellulose	58·81	48·99	59·39	54·14
Lignin	21·51	15·66	20·30	17·30
Pentosans in cellulose	15·79	11·26	14·63	12·24
Pentosans not in cellulose	8·71	8·13	10·15	10·16
Methoxyl content	5·80	4·05	5·50	4·69

comparative purposes a lignin determination was applied to a small sample of decayed wood taken from the immediate vicinity of the white patch. 0·3408 g. of decayed wood contained 0·0698 g. or 20·48 % of lignin. While the white material was loosely packed together, the surrounding pale brown material was relatively compact, so that a complete separation was possible. During drying, the intensely white colour of the former was observed to change gradually to a pale brown of lighter tint than that of the latter.

Discussion of results.

It is generally accepted that *Polyporus hispidus* and *Stereum hirsutum* cause white rot in wood in the sense that the decayed wood assumes a lighter colour than the original sound wood, and that frequently the decayed material contains patches or pockets of a white material resembling cellulose in

appearance. This opinion has been confirmed in the experiments described above and definite white patches were observed in the case of the decay of oak sapwood by *Stereum hirsutum*. There appears to be some doubt, however, as to whether the decay of wood by *Armillaria mellea* can truly be described as a white rot. In this case the decayed wood was not markedly lighter in colour than the sound wood, despite the fact that Hubert [1924] has described the decay as a white rot. The data in Table I reveal however, that, at the stage of decomposition examined, *A. mellea* causes a type of decay whose chemical effect in wood has not hitherto been described in detail. It can be seen that after 16 months' decay the loss of 8·66 % of the oven-dry weight of the original wood is almost wholly accounted for by cellulose depletion. Of the pentosans, only those associated with the cellulose are attacked, and lignin appears to have been unaffected by the fungus. Thus far, the decay bears a close resemblance in effect to the several brown rots which have been described from time to time, but as brown rots in general have been shown to cause a marked increase in the alkali-solubility of the residual wood substance, the decay caused by *A. mellea* cannot be classified with them, for in this case no marked increase in alkali-solubility of the wood residue takes place. Undoubtedly had part of the water-soluble material not been removed from the decayed wood its alkali-solubility would have been greater than that recorded in Table I, but, at the same time, a tendency is here disclosed towards the reduction of the total alkali-solubility of the major constituents as decay proceeds. The same tendency has been observed in the white rot caused by *Polystictus versicolor* [Campbell, 1930] and the analytical data (Table I) reveal that its cause is to be sought in the marked reduction during decay in the alkali-solubility of the pentosans not in the cellulose. Thus even in the absence of analytical data to show whether lignin is in fact decomposed at a later stage, it is apparent that at the stage examined, decay by *A. mellea* possesses chemical characteristics of both brown and white rots, but that in view of the effect on the alkali-solubility of the decayed residue, it may, with reason, be described as a variety of white rot.

With regard to the respective effects on wood substance of *Stereum hirsutum* and *Polyporus hispidus*, it can be seen from the data in Table II that these are essentially the same, despite the fact that in the former case the loss in weight due to decay is approximately twice as great as in the latter. Lignin is decomposed, but in each case by far the greater proportion of the total loss in weight is accounted for by the depletion of the cellulose and its associated pentosans. The percentage amount of pentosans not in the cellulose remains practically constant throughout the decay, and the fall in the methoxyl content is much less than that of the lignin content. Finally, this type of decay does not cause a marked increase in the alkali-solubility of the principal components of wood substance, and it is probable that this fact can be ascribed to the same causes which are operative in the decay of wood by *Polystictus versicolor* and *Armillaria mellea*.

Consideration of the chemical composition of the patches of white material produced during the decay of oak sapwood by Stereum hirsutum.

It has long been considered by biologists that in certain white rots of wood, where patches or pockets of a white material are found to occur, the fungi concerned have a selective action on lignin, and that the white residue consists entirely of cellulose. This conclusion has been based primarily on the reaction of this material to the so-called cellulose stains, but, so far as is known, no purely chemical test has hitherto been applied to it. This has undoubtedly been due to the fact that the white patches in question are usually small, and that the isolation of samples sufficiently large for analysis has been a matter of difficulty. In the experiments recorded above, the samples were too small for more than one analytical determination in each case, but it was considered that lignin determinations would be comparatively easy to apply, and that, in spite of some degree of experimental error, they would provide a definite answer to the questions as to whether the white patch consisted entirely of cellulose, and whether the fungus had had a marked selective action on lignin.

In the first place, it is apparent that the white material still contains lignin, although its percentage amount cannot be directly compared with the lignin content of the original wood, since the loss in weight due to decay is not known. It may safely be deduced indirectly however that the portion of the original wood which gave rise to the white material must have lost considerably more than 20 % of its oven-dry weight before this residue attained a lignin content of 12·5 %, and, since the lignin content of the original wood (Table II) was 21·51 %, it is obvious that wood components other than lignin—and of these chiefly the cellulose—must have been decomposed. From the fact that the lignin content of the material immediately contiguous to the white patch is approximately the same as the actual lignin content of the decayed wood in Table II, it must be deduced that decay has not been confined to the material which ultimately became white.

Conclusions and summary.

From the above considerations it is to be concluded that the three examples of white rots examined have only one feature in common, and that this is shared by the white rot caused by *Polystictus versicolor*. In every case there is no marked increase in the total alkali-solubility of the major components of wood substance and in the case of *Polystictus versicolor* and *Armillaria mellea* this has been proved to be due to the fact that one component—namely the pentosans not in the cellulose—becomes less soluble in 1 % NaOH as decay proceeds. The white rots in question are thus sharply differentiated from the brown rots, for it has been found in some of the latter, that even in very advanced stages of decay the residual wood substance is much more soluble in alkali than the original sound wood. While it has been established

that the detailed chemical effect on wood substance of both *Polyporus hispidus* and *Stereum hirsutum* is essentially similar in all respects, this effect can in turn be differentiated from that of *Polystictus versicolor* when the manner in which pentosans are decomposed is considered. *Polystictus versicolor* attacks the pentosans not in the cellulose to a greater extent than the pentosans which are associated with the cellulose, whereas *Polyporus hispidus* and *Stereum hirsutum* attack the pentosans in the cellulose and have little or no effect on the pentosans not in the cellulose, at the stages of decay which were examined. In view of the effect on the alkali-solubility of wood substance of the decay caused by *Armillaria mellea*, and the fact that the decayed wood possesses to some extent the visual characteristics of a white rot, it must be concluded that in this type of rot a stage can exist in which lignin is apparently unaffected by the fungus.

In the case of the "pocket" rot caused by *Stereum hirsutum*, it is to be concluded that this fungus cannot be credited with a selective action on lignin. The white patches referred to above represent localities in a decaying mass in which decomposition has progressed to a greater extent than in the surrounding material. In these localities cellulose is decomposed as well as lignin, and even after the development of the white colour some lignin remains.

The author desires to express his indebtedness to Messrs K. St G. Cartwright and W. P. K. Findlay for carrying out the inoculations, to Sir James Irvine for criticising the manuscript, and to Mr R. S. Pearson, Director, Forest Products Research, for permission to publish these results.

REFERENCES.

Campbell (1930). *Biochem. J.* **24**, 1235.
Falck (1927). *Ber. deutsch. chem. Ges.* **60**, 225.
Hubert (1924). *J. Agric. Res.* **29**, 551.

CCXXI. FURTHER OBSERVATIONS ON THE OXIDATION OF LACTIC ACID BY BRAIN TISSUE.

By CHARLES AMOS ASHFORD[1]
and ERIC GORDON HOLMES.

From the Biochemical and Pharmacological Laboratories, Cambridge.

(*Received October 30th, 1931.*)

In an earlier communication [Holmes and Ashford, 1930], we reported experiments bearing upon the question of the "Meyerhof cycle" in brain tissue. If lactate is added to brain tissue *in vitro*, and the system thoroughly oxygenated by shaking in a Barcroft's apparatus, or otherwise, it is easy to show that the amount of such added lactate which disappears is far greater than can be accounted for by the additional oxygen taken up by reason of the presence of the lactate. The relationship $\frac{\text{extra lactic acid disappearing}}{\text{lactic acid equivalent of extra } O_2}$ (the "Meyerhof quotient") has a value of about 3·0. We did not succeed in demonstrating any synthesis of carbohydrate; and such a synthesis is required by the hypothesis advanced by Meyerhof, Warburg and their collaborators.

In this paper, experiments are described which substantiate the validity of our previous findings and amplify them in certain important particulars. In the first place, the experiments reported in our first communication on this subject were all performed in a medium which was buffered by phosphate. The experiments have been repeated, therefore, using the CO_2-bicarbonate buffer, but avoiding the manometric method of Warburg, which involves working with very small amounts of tissue. We have investigated more fully the respiratory quotient of the tissue, both in the presence and in the absence of added lactate, and we have endeavoured to trace the fate of the added lactate which disappears.

Section I.

Experiments carried out with bicarbonate-CO_2 buffers.

Since we desired to follow the disappearance of lactic acid by chemical, and not by indirect manometric methods, it was necessary for us to work on a fairly large scale, and to employ at least 2 g. of tissue. At the suggestion of Prof. J. B. S. Haldane, we decided to estimate the oxygen consumption by means of the gas analysis technique.

[1] Beit Memorial Research Fellow.

For this purpose two flasks with wide necks, and of approximately 50 cc. capacity were obtained and arranged as shown in Fig. 1. The flask and attachments were carried on a wooden carriage, which was so shaped that it could be clamped into the mounting of the Barcroft thermostat and shaken like an ordinary Barcroft manometer. The neck of the flask was marked with a diamond at the point reached by the lower end of the rubber bung, and the volume of the whole apparatus, with tubes in place, accurately determined. The apparatus was made and used in duplicate.

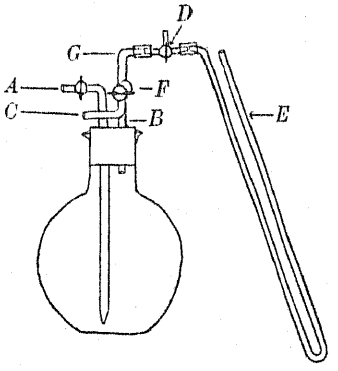


Fig. 1. Diagram (not to scale) of flask with stopper in place. Wooden carriage omitted. *A*. Gas entrance tube. *B*. Tube through which gas escapes during filling, and through which samples are subsequently withdrawn. *C*. Tube through which mercury is expelled during taking of sample. *D*. Three-way tap by means of which flask may be connected to air or manometer. *E*. Mercury manometer. *F*. Two-way tap enabling *B* or *C* to be connected to *G*. All glass tubes and taps are of 1 mm. capillary tubing.

The experimental procedure was as follows.

2 g. of chopped (rabbit's) brain tissue were weighed out, and placed in each flask. 5·0 cc. of Ringer's solution containing 0·025 *M* sodium bicarbonate [Warburg, 1926] was placed in one flask (*OP*) and 5 cc. of a similar bicarbonate Ringer, containing in addition 0·5 % lithium lactate, was added to the other (*OL*). The mass of tissue was broken up with forceps, the stoppers put in place, and the bottles were placed in the carriages. About 4 litres of a CO_2/O_2 (approx. 5 % CO_2 in O_2) gas mixture were bubbled through both bottles, which were arranged in series. Tap *A* was then closed, tap *D* turned so as to connect the bottles to the manometers, and the bottles were placed in the thermostat at 37° and shaken for a few minutes, by which time the manometers had become steady. Tap *D* was then opened to the air for a few moments; taps *D* and *F* were then closed, and the apparatus was shaken at 37° for the required time, usually for 3 hours.

At the end of this period, the bottles were removed, and placed, in their carriages, in a tank of water at room temperature which was stirred by a brisk stream of air bubbles. The manometers were connected to the bottles by turning taps *F* and *D*, and readings of the pressure inside the bottles were taken at intervals, until the manometers became steady. Tap *F* was then closed, and the bottles were removed from their carriages, and immersed in a tall jar of water. They were connected with gas-sampling tubes filled with mercury, and samples withdrawn in the usual way, the dead space in *G* and the connecting tubes being filled with mercury by gravity, the mercury escaping through *C*. The samples were then analysed in a Haldane apparatus [Haldane, 1920], by the standard technique for oxygen-rich mixtures (*i.e.* after preliminary dilution with nitrogen in the gas burette).

In this way sufficient data were obtained to calculate the initial and final volumes of dry gas at N.T.P., and the initial and final percentage composition.

The initial percentage composition was determined in a sample taken direct from the aspirator, where the gas mixture was stored over saturated $CaCl_2$, immediately after the bottles had been filled.

The initial volumes were those of the bottles (already determined) corrected for barometric pressure, temperature (37°), and the tension of aqueous vapour at 37°. The final volumes were those of the bottles, with the following corrections.

(1) Temperature (that of the tank in which they were immersed after removal from the thermostat).

(2) Tension of aqueous vapour at that temperature.

(3) Pressure. This was that of the barometer, *minus* the pressure difference recorded by the manometer. To obtain the latter, the manometer reading had to be multiplied by $\cos\theta$ where θ was the angle of inclination of the manometer to the vertical.

No correction was made for the solubility of O_2 in water at room temperature.

From these data, the amounts of oxygen and CO_2 present in the gas phase in the bottles at the beginning and end of the experiment were calculated, the difference in the values found for oxygen representing the oxygen consumption of the tissues.

Immediately after the final gas samples had been taken, tap *A* was opened, and the stoppers were removed from the bottles. The tubes were washed with the precipitating reagent, and the tissue was worked up for chemical estimation by one of the methods described in our previous paper [Holmes and Ashford, 1930]. For lactic acid only, trichloroacetic acid was employed, for "free sugar," dilute alcohol, for glycogen, absolute alcohol. Two points only need mention. (*a*) During evaporation of the alcohol, CO_2 escapes and the fluid tends to become strongly alkaline, on account of the breakdown of the bicarbonate to carbonate. To guard against this, a few drops of bromocresol purple were added, and the fluid was kept neutral by the addition of dilute HCl in alcohol, drop by drop as required, during evaporation. (*b*) The calculation of the "extra" lactic acid removed in these experiments was made as follows.

If the initial lactic acid of the tissue = *IP*, and the amount of lactic acid added = *L*, and if after oxygenation, for tissue alone, lactic acid remaining = *OP* and for tissue with lactic addition, lactic acid remaining = *OL*, then lactic removed from plain sample = $IP - OP$.

Lactic acid removed from sample to which lactic acid had been added $= (L + IP - OL)$.

$$\text{Therefore extra lactic removed} = (L + IP - OL) - (IP - OP)$$
$$= L - OL + OP.$$

Hence the estimation of the initial lactic acid of the tissue was unnecessary, and its omission afforded both a saving of time and tissue. *L*, *OP* and *OL* were estimated chemically.

Table I.

Exp.	Ringer	Initial CO_2 %	p_H Initial	p_H Final	Extra lactic acid removed (mg.)	O_2 consumed (mm.³) Plain	O_2 consumed (mm.³) Lactate	Extra O_2	Lactic equiv. of extra O_2	"Q"	Expected synthesis	Observed synth. (mg.) Free carb.	Observed synth. (mg.) Glycogen	Duration of exp. (min.)	Remarks
14. xi. 30	Bicarb.	5·04	7·20	—	8·54	6070	10670	4600	5·95	1·43	3·6	—	—	240	Oxygen
17. xi. 30	,,	4·83	7·21	—	6·11	5670	7990	2320	2·95	2·1	3·2	—	—	245	Oxygen
21. xi. 30	,,	5·51	7·16	—	10·17	5230	10950	4720	6·37	1·6	3·8	—	—	240	Oxygen
24. xi. 30	Phosph.	0	7·6	—	7·51	4080	5080	1000	1·34	5·6	6·7	—	—	225	Air } Parallel exps.
	,,	0	7·6	—	12·71	4580	8700	4120	5·55	2·3	7·2	—	—	225	Oxygen }
26. xi. 30	Bicarb.	5·03	7·2	—	9·40	6020	8380	2360	3·17	2·9	6·2	—	—	180	Oxygen } Parallel
	Phosph.	0	7·7	—	11·11	4000	6720	2720	3·66	3·0	7·4	—	—	180	Oxygen, Barcroft's } exps.
28. xi. 30	Bicarb.	4·45	7·24	7·29	12·14	4970	8310	3340	4·50	2·7	7·6	0·140	—	180	Oxygen
3. xii. 30	,,	6·54	7·11	—	6·68	5920	8420	2500	3·36	2·0	3·3	0·031	—	180	Oxygen } Parallel exps
	Phosph.	0	7·18	—	8·37	3900	6300	1400	1·88	4·4	6·5	0·120	—	180	Oxygen }
6. xii. 30	Bicarb.	5·38	7·22	7·12	7·74	5270	8720	3450	4·65	1·7	3·1	0·190	—	180	Oxygen
12. xii. 30	,,	5·03	7·14	7·09	—	6860	8990	2130	2·87	—	—	—	−0·22	195	Oxygen. Sodium lactate 1 %
18. xii. 30	,,	6·33	7·15	7·20	5·34	5150	8120	2970	4·00	1·5	1·9	—	+0·12	180	Oxygen. Sodium lactate 0·5 %
15. i. 31	,,	7·45	7·01	—	11·75	—	—	—	—	—	—	—	−0·15	180	Oxygen. Sodium lactate 0·5 %
14. iv. 31	Phosph.	—	7·4	—	11·8	3680	6220	2540	3·41	3·5	8·4	—	—	,,	Oxygen, Barcroft's
17. iv. 31	,,	—	7·4	—	10·0	4100	6425	2325	3·13	3·2	6·8	—	—	,,	Oxygen, Barcroft's
24. iv. 31	,,	—	7·4	—	9·15	4040	6590	2550	3·38	2·7	5·8	—	—	,,	Oxygen, Barcroft's
14. v. 31	,,	—	7·4	—	9·75	4145	6675	2530	3·40	2·9	6·3	—	—	,,	Oxygen, Barcroft's
3. vi. 31	,,	—	7·4	—	10·5	4290	6370	2080	2·80	3·76	7·7	—	—	,,	Oxygen, Barcroft's
13. vi. 31	,,	—	7·4	—	8·14	1460	3710	2250	3·00	2·7	5·1	—	—	,,	Oxygen, Barcroft's. Insulin exp., 1 g. tissue
18. vi. 31*	,,	—	7·4	—	5·5	1510	3000	1490	2·00	2·7	3·5	—	—	135	Oxygen, Barcroft's. Insulin exp., 1 g. tissue

* Initial lactic acid content of tissue = 0·47 mg. per g. Except where otherwise stated, 0·5 % lithium lactate was used.
p_H by indicator method in all experiments with phosphate buffer.

Table I shows the results obtained in a number of experiments. The differences between the initial and final amounts of oxygen were large, and the technique, as a whole, proved satisfactory.

Several important points emerge from inspection of Table I. In the first place, it will be seen that the "quotient" is rather lower (average 2) when the Ringer is buffered by bicarbonate than when it is buffered by phosphate (average 3); the oxygen uptake is distinctly larger both in the presence and

Table II.

Average O_2 uptake of 2 g. of tissue in 3 hours at 37°, with different buffers and gas mixtures

Conditions	Oxygen uptake in mm.^{3}		
	Plain	Lactate	Extra
Air/PO_4	3520	4205	685
Air/CO_2/bicarb.	3570	4420	850
O_2/PO_4	3940	6430	2490
O_2/CO_2/bicarb.	5700	8490	2790

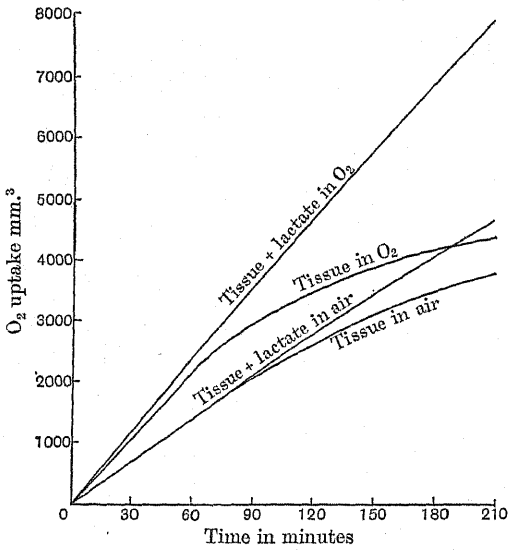


Fig. 2. Effect of oxygen tension on oxygen uptake of brain tissue. All 2 g. tissue. PO_4–Ringer p_H 7·4 at 37°. Lactate 0·5 %.

in the absence of lactate, than when a phosphate buffer is used. Our original experiments were performed in air; and when air is replaced by oxygen, even with phosphate buffer, there is a considerable increase in oxygen consumption (see Fig. 2); nevertheless tissue in bicarbonate buffer and CO_2/O_2 gas mixture shows a still larger uptake than tissue in O_2 and phosphate buffer (see Table II and Fig. 2). In spite of the fall in the quotient in the experiments with O_2/CO_2 and $NaHCO_3$, it will be seen that the net result is that the expected synthesis of carbohydrate is absolutely larger in both sets of experiments carried out in oxygen as compared with those carried out in air. Table II

summarises the differences in oxygen uptake occurring under the different conditions just discussed.

The data given in some cases for "initial" and "final" p_H in Table I, were obtained in order to be sure that, in spite of the large changes in CO_2 percentage which occurred during the experiment (*e.g.* initial 5 %, final 10 %), there was no serious change in p_H.

Table III. *Oxygen uptake of different amounts of chopped brain tissue, in oxygen and Ringer buffered with phosphate* p_H *7·4.*

Amount of tissue taken g.	O_2 uptake per g. in 3 hours mm.^{3}
0·3	2180
0·5	2040
1·0	2025
1·5	2390 (single exp.)
2·0	1970

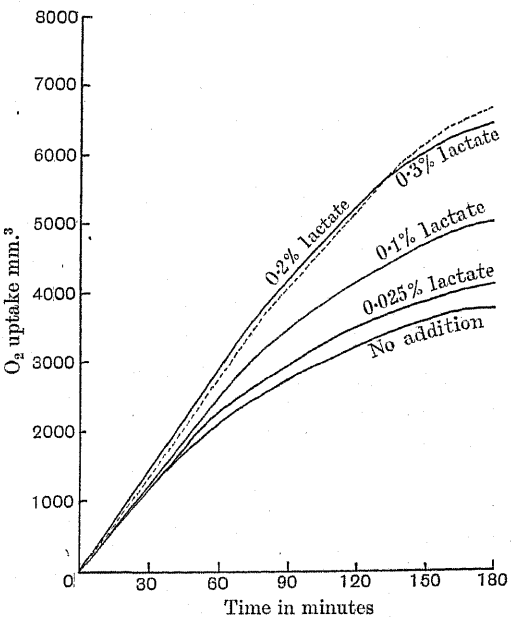


Fig. 3. Effect of varying lactate concentration. 2 g. tissue. p_H 7·4, in O_2 at 37°.

There is no difficulty about determining the initial p_H by means of the quinhydrone electrode. The final p_H was determined in a duplicate flask provided with the necessary electrodes and a device for introducing the quinhydrone at the conclusion of the experiment.

It seems to us that these experiments show that neither the existence of the "quotient" nor the absence of synthesis can be ascribed either to failure of adequate oxygen supply, or to buffering with phosphate, since both phenomena occur equally in air and in oxygen, and in a bicarbonate/CO_2 buffered medium as well as in one buffered with phosphate. Dixon and Elliott [1929]

point out that, if the availability of oxygen is not a limiting factor in experiments with the Barcroft apparatus, then the relationship between oxygen uptake and weight of tissue used will be linear, or, put in another way, the uptake per g. of tissue will be constant, no matter how much tissue is employed in any given experiment. Table III shows that this is the case in our experiments.

One further point should be mentioned here. In most of the earlier experiments, an arbitrary concentration of lithium lactate of 0·5 % was used. Figs. 3 and 4 show the effect of different concentrations of this salt from 0·025 % to 0·8 %. It will be seen that little increase in oxygen uptake is

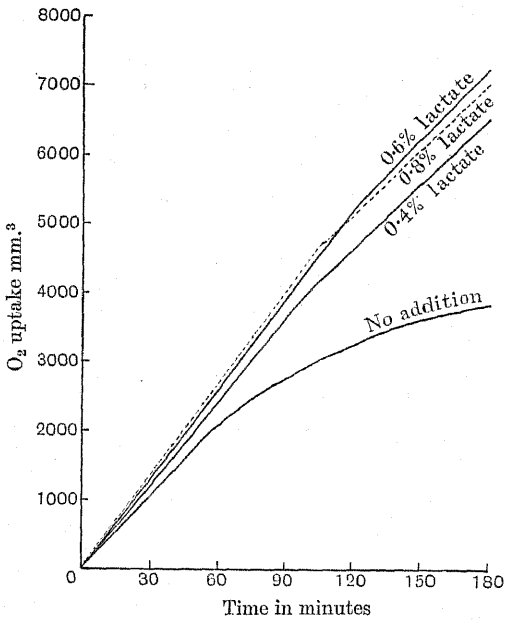


Fig. 4. Varying lactate concentration. 2 g. tissue; p_H 7·4; in O_2 at 37°.

obtained by increasing above 0·4 %. 0·5 % has accordingly been used in all subsequent experiments for convenience of estimation. At lower concentrations, the oxygen uptake curve soon falls off although the initial velocity is in all cases the same. Table IV shows the Meyerhof quotient under different conditions of lactate concentration. It will be seen that at lower concentrations the quotient is somewhat lower, but between 0·3 % and 0·8 % it has an average value of 2·7 to 2·8. These experiments have a further significance in connection with the question of the "sparing" of substrates to be referred to later.

A question of obvious importance is that of the effect of the chopping of brain tissue on its oxidative mechanisms. It has previously been reported by one of us [Holmes, 1930] that the oxygen consumptions of chopped and sliced rabbit brain are about equal. Nevertheless, we wished to try to obtain some

more positive data about the effect of chopping. It was out of the question to use intact mammalian brain (as a control to the chopped tissue), for clearly O_2 could not possibly diffuse quickly enough to supply its needs. On the other hand, with frog's brain in oxygen, and at room temperature, it was to be expected that at the lower temperature the chemical processes of oxidation

Table IV. *Effect of the lactate concentration on the Meyerhof quotient.*

In all experiments 2 g. of tissue were used in O_2 at p_H 7·4 and 37° (Ringer buffered with phosphate).

Concentration of lithium lactate %	Meyerhof quotient
0·025	1·93
0·05	2·26
0·1	1·78
0·2	2·15
0·3	2·54
0·4	2·82
0·5	2·71
0·6	2·96
0·8	2·80

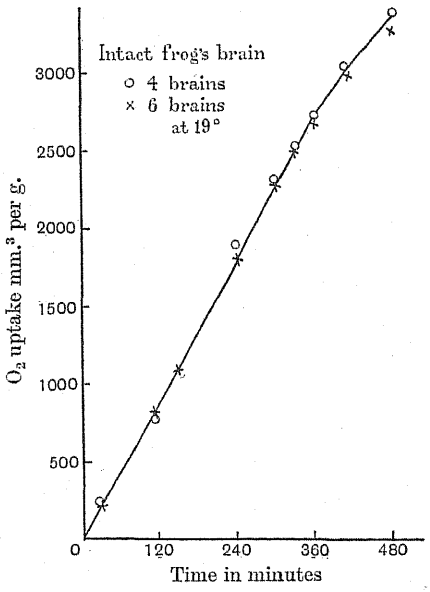


Fig. 5. Oxygen uptake of intact frog's brain at 19°. In O_2 at p_H 7·4.

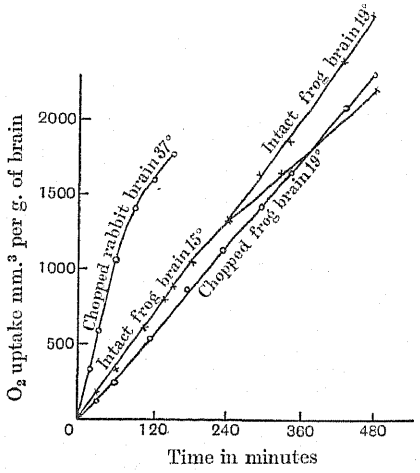


Fig. 6. Oxygen uptake of intact and chopped frog's brain.

would be retarded far more than the physical process of diffusion. We therefore performed some experiments in which the oxygen uptake of intact, freshly dissected frog's brain was determined in the small type of Barcroft apparatus, which is calibrated for 1·0 cc. of fluid. An intact frog's brain is larger than the individual fragments of chopped rabbit's brain used in the other experiments.

Fig. 5 shows the results of two experiments showing that the oxygen uptake per g. is the same when different amounts of tissue are used (4 brains in the one case, 6 in the other).

Fig. 6 shows the effect of chopping on the uptake of frog's brain. The total uptake of the chopped brain is some 16 % less than that of the intact brain, which, in our view, does not suggest that the chopping is likely to have had any very serious deleterious effect upon the oxidative mechanisms. This stands in contrast with the observation of Meyerhof [1919] that chopping of frog's muscle leads to a large increase in the oxygen uptake as compared with the intact muscle.

For purposes of comparison there is added a curve showing the uptake per g. of chopped rabbit brain at 37°.

We think that these experiments tend to show—admittedly, they do not conclusively prove—that our practice of chopping brain tissue is unlikely to interfere with its normal oxidative processes. They do not, of course, throw any light on the possibility of damage to other mechanisms. It must be remembered that less damage is likely to be caused to individual cells in chopping brain, than would be caused by chopping muscle, the long fibres of which would inevitably suffer. It has been shown [Holmes, 1930] that most of the O_2 uptake of brain is due to the cells, and not to the axons.

Section II.

Fate of the lactic acid.

Since the lactic acid which disappeared, over and above that which was accounted for by the "extra" oxygen, was not synthesised to glycogen, or to any form of reducing carbohydrate which could be estimated by the usual methods, we endeavoured, by other means, to trace its fate.

During an informal discussion of our results, it was suggested by Dr B. Woolf, as a possible explanation of them, that all the lactic acid was in reality being oxidised, but that part of it was "sparing" the oxidation of some other substance present in the brain tissue. This would, of course, account for the fact that the "extra" oxygen as measured by the difference between *OL* and *OP* was too small to account for the whole of the lactate which disappeared, simply because other materials were oxidised in *OP* which in *OL* were "spared" and so required no oxygen at all. This suggestion is clearly much to the point; it is also one which it is extremely difficult either to substantiate or to disprove.

It seems clear, however, that it cannot afford the correct explanation, if, in any circumstances, it can be shown that the total lactic acid disappearing would require, if completely oxidised, more oxygen than the total amount of that gas taken up in the manometer containing tissue *plus* added lactate (*OL*). In most of our experiments this is not the case, though, in a good many, there is only a comparatively small margin between the two amounts. If,

however, the experiments are carried out on tissue which has been depleted of its own substrates, either by washing, or by exposure to oxygen, or by the preliminary administration of insulin to the animal, then the oxygen equivalent of the total amount of lactic acid disappearing is very considerably greater than the total O_2 uptake of *OL*. Experiments of this nature are detailed in Table V, and it seems to us therefore unlikely that our results are due simply to the "sparing" of other substrates by lactic acid.

Table V.

Date	O_2 equivalent of total lactic removed mm.^{3}	Total O_2 taken up by *OL* mm.^{3}	Remarks
21. viii. 30	3420	2770	Washed tissue
22. viii. 30	3870	2440	Washed tissue
26. viii. 30	1640	751	Tissue washed and kept 12 hrs. in ice-chest
29. vi. 31	4350	2800	Tissue shaken in O_2 for 3 hrs. before lactate added and readings commenced
1. vii. 31	3185	1564	Tissue shaken in O_2 for 3½ hrs. before lactate added and readings commenced
18. v. 31	4290	3000	Animal rendered hypoglycaemic with insulin before killing
13. v. 31	6360	3710	Animal hypoglycaemic with insulin before killing

If the concentration of lactate is sufficiently low, this relationship does not hold good, at least in the case of washed brain; probably because the enzymes concerned are then no longer saturated with substrate. This point is further discussed at a later stage.

In endeavouring to trace the fate of the lactic acid, it seemed to us to be best to obtain information about the respiratory quotient of the tissue, and particularly about that of the "extra" oxygen. Some preliminary experiments on this point were reported in our first communication [1930]. The technique finally adopted was the one of which the details are given by Dixon and Elliott [1929]. For our purposes, six Barcroft manometers were required, and we have used either the ordinary (3 cc.) or the large type. We may designate them *A*, *B*, *C*, *D*, *E*, *F*. *A* contains tissue and plain Ringer, and the CO_2 is absorbed by sodium hydroxide. *B* is similar to *A*, but contains lactate Ringer. *C* and *E* each contain tissue and plain Ringer, but instead of sodium hydroxide they have a Keilin cup containing saturated oxalic acid solution hung on the sodium hydroxide pot. *D* and *F* are arranged similarly to *C* and *E*, but contain lactate Ringer instead of plain Ringer. All the cups were filled with oxygen.

C and *D* were put into the thermostat and the taps closed as soon as temperature equilibrium was obtained. The acid was immediately spilled into the body of the cup by sharp shaking, and the manometers read until the reading became steady; this took usually 5 to 10 minutes. These readings represented preformed CO_2 in *OL* and *OP* respectively.

The remaining four Barcroft apparatus were then shaken in the thermostat for an appropriate period—2 to 3 hours. The manometers of *A* and *B* were read, and the difference between them gave the "extra" oxygen. At the end of the period, the acid in the cups of *E* and *F* was spilled, and the manometer reading represented the difference between O_2 absorption and total CO_2 in the system at the end of the experiment. Thus, if, for the moment, we take the letters to represent the gas volumes indicated by the readings of the various manometers (*i.e.* the manometer readings × factor)

$A - E = CO_2$ contained in *OP*,
$B - F = CO_2$ contained in *OL*,
$A - E - C = CO_2$ evolved by *OP* (*i.e.* contained in *OP minus* initial CO_2),
$B - F - D = CO_2$ evolved by *OL*.

From the data so obtained, we can calculate the respiratory quotient of the tissue alone, the tissue to which lactate has been added, and the amount of CO_2 corresponding to the "extra" oxygen.

Table VI gives some results obtained by this method. It will be seen that it includes certain experiments performed on the brains of animals with insulin hypoglycaemia. These were performed with the object of discovering whether there was any definite fall in the respiratory quotient as a result of the shortage of lactic acid which has been shown [Holmes and Holmes, 1925] to exist in the brain under these circumstances. No such fall is apparent: evidently, therefore, substrates are still present which maintain the R.Q. at, or close to, unity.

Table VI. *Tables of values found for respiratory quotients.*

Experiments in air				Experiments in O_2			
Date	Tissue only	Extra O_2		Date	Tissue only	Extra O_2	
8. vii. 30	0·935	0·937	Normal animals	8. v. 31	0·924	0·96	Normal animals
11. vii. 30	0·985	0·920	,,	9. v. 31	0·87	—	,,
31. vii. 30	0·758	1·124	,,	12. v. 31	0·87	1·10	,,
5. viii. 30	0·875	1·128	,,	13. v. 31	0·96	0·94	Insulin animals
7. viii. 30	0·909	1·020	,,	18. v. 31	0·89	0·96	,,
20. viii. 30	0·928	0·984	,,				
Mean	0·912	1·018					

The R.Q. of the "extra" O_2 is 1·0 (mean values, 1·018 and 1·03, are certainly within the limits of experimental error) whether the experiments are performed in O_2 or in air. Loebel [1925] gives figures which are somewhat lower than these. This, on the face of it, suggests that the extra oxygen is concerned with the complete oxidation of a corresponding amount of lactic acid. We realise that conclusions from experiments of this kind must be drawn with caution; nevertheless, these findings make it highly improbable that the existence of the Meyerhof quotient depends on a partial oxidation such as the conversion of lactic acid to pyruvic acid, since such a process would give a Meyerhof quotient of 6·0, but R.Q. of the extra oxygen would be 0. The results

obtained in the respiratory quotient experiments could be explained if lactic acid were changed to acetic acid. This would involve the absorption of one molecule of O_2, and the formation of one molecule of CO_2. Apparently, however, this change does not take place, for if it did, either the acetic acid must be further oxidised, or must remain behind unchanged. Brain tissue, however, takes up no extra oxygen with acetate, nor does acetate accelerate the reduction of methylene blue by brain tissue (Thunberg technique); while, again, we were unable to demonstrate an excess of volatile acid in *OL* at the end of the experiment, under conditions in which acetic acid could be quantitatively recovered. Tests for pyruvic acid and acetaldehyde were negative.

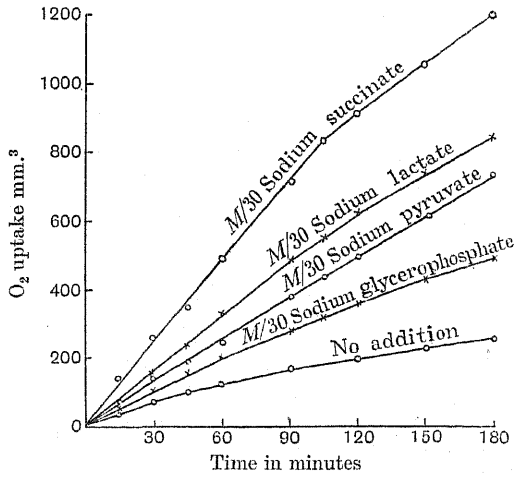


Fig. 7. 0·3 g. tissue in 3 cc. of fluid; p_H 7·4; in air at 37°.

This was again demonstrated by a large scale experiment in which 90 g. of pig's brain were oxygenated, both in the presence and absence of added lactate. The tissues, and also a similar initial sample, were worked up with trichloroacetic acid and filtered. The filtrates were neutralised and concentrated *in vacuo* in the presence of sodium sulphite [*cf.* Neuberg and Kobel, 1930; Case and Cook, 1931]. In no case was a precipitate obtained on adding 2:4-dinitrophenylhydrazine in hydrochloric acid.

In such attempts to follow the course of the disappearance of the lactic acid, the fate of a number of substrates which might possibly act as intermediaries has been investigated, both by the Barcroft aerobic technique and the Thunberg methylene blue reduction technique. Toennissen and Brinkmann [1930] have put forward a scheme involving, for the oxidation of lactic acid by muscle, the formation of pyruvic acid, which in turn gives an unstable 6-carbon compound by the loss of hydrogen. This, in turn, gives succinic and formic acids, which under appropriate conditions may be oxidised. It has already been mentioned that no evidence of pyruvic acid formation has been

obtained in our experiments. In one experiment conducted at room temperature, as opposed to those at 37°, there was very faint and doubtful evidence of the presence of pyruvic acid, as shown by the nitroprusside reaction. In spite of this, we have performed experiments with substrates suggested by Toennissen's work, using equivalent concentrations of each; Fig. 7 shows the oxygen uptake of brain with different substrates. It will be seen that succinate [*cf.* Quastel and Wheatley, 1931], lactate, pyruvate and glycerophosphate are all "oxidised." On the other hand, no evidence of an extra oxygen uptake was obtained with either formate or acetate while mandelate actually caused an inhibition of the order of 50 %. A parallel series of findings is obtained with the methylene blue technique (Table VII).

Table VII. *Effect of various substrates on reduction of methylene blue by brain tissue.*

Hydrogen donators		Not hydrogen donators	
Lactate, Glucose, Succinate, Glycerophosphate	Approx. the same activity	Glycerol, Dihydroxyacetone	No change in reduction time
Pyruvate	Slight change in reduction time	Acetate, Formate, Mandelate	inhibitors

Since our attempts to discover the fate of the lactic acid by qualitative tests for likely substances have given negative results, and since our determinations of the respiratory quotient threw no light on the probable fate of that portion of it which had not been oxidised, we decided, on the advice of Miss M. Stephenson, to perform estimations of total "soluble" carbon. If we estimated the total carbon in protein- and lipoid-free filtrates of *OL* and *OP*, then we should expect that the carbon in *OL* would be equal to that in *OP*, *plus* the carbon added as lactate, *minus* that which had been oxidised to CO_2, and absorbed by the potash during the experiment. If this were not the case, then either the lactate must have been changed to some substance which, though not glycogen, was still not soluble in the precipitating agent used in the preparation of the filtrates, or, alternatively, there must have been some fallacy inherent in our previous experiments or in our reasoning. The method adopted for the estimation of total carbon was the "wet combustion" method of Raistrick [1931]. It was necessary to choose a reagent for precipitating protein and lipoid which was free from carbon; while the presence of halogens in it was undesirable. The acid mercuric sulphate reagent of Hopkins and Cole (10 % mercuric sulphate in 7 % sulphuric acid) was found to be suitable.

2 g. of chopped brain tissue were put into the right-hand cups of each of four large Barcroft manometers, two of which contained plain Ringer, and the other two lactate Ringer. The Ringer in both cases was buffered with phosphate. Each apparatus was filled with oxygen, and shaken for 3 hours in the thermostat, the oxygen uptake being observed as usual. At the end of the experiment the 4 g. of tissue (from the two *OP* Barcroft's) were washed

into a 50 cc. volumetric flask with 10 cc. of the mercuric sulphate reagent, and the necessary amount of water to make up to volume. The 4 g. of *OL* were similarly treated. After standing for not less than 1 hour, the contents of the flasks were transferred to centrifuge-tubes and centrifuged. The supernatant fluid was poured off, and the mercury removed with H_2S. HgS was removed by filtration on a small Büchner funnel, then through an ordinary dry filter-paper, and H_2S by aeration with moist air. 5 cc. of the solution were taken for estimation, and represented one-tenth of the original amount of material.

Table VIII shows the results obtained in these experiments. The figures in the column labelled "calculated" were obtained by adding to the value for *OP* found by estimation the difference between the amount of carbon in the added lactate and that in the CO_2 corresponding to the extra oxygen; in other words, by adding to the value for *OP* the carbon of the "expected synthesis."

Table VIII.

Date	Carbon added as lactate mg.	Carbon equiv. of extra O_2 mg.	Carbon found in *OP* mg.	Carbon calculated in *OL* mg.	Carbon found in *OL* mg.	Percentage difference
25. v. 31	16·16	2·12	13·99	28·03	27·04	3·6
3. vi. 31	16·64	2·24	12·85	27·25	24·65	9·5
14. v. 31	9·91	2·73	12·78	22·69	22·42	1·2
9. vi. 31	16·58	2·78	13·75	27·55	25·89	6·0
					Average	5·07

It will be observed that the values for carbon found in *OL* are less on the average by some 5 % than those "calculated." This may be the result of experimental error; the figures for the calculated value depend upon three separate measurements; (i) the estimation of the added lactate, (ii) the measurement of the "extra" oxygen, and (iii) the estimation of the carbon in *OP*, and some error is likely to creep in. It is, however, possible—since the discrepancy is always in one direction—that it really means that the fate of a small amount of the added lactate is in some way different from that of the remainder.

We feel that these experiments give an assurance that we are dealing with a real phenomenon. We have already stated our reasons for thinking that we are not dealing simply with the sparing of some other substrate by lactic acid. The carbon estimations do not throw any special light on the question of the sparing of substances soluble in mercuric sulphate, as there is clearly no way of identifying the carbon as being derived from lactic acid. But they seem to us to provide some additional evidence against the sparing of protein or of lipoids. If protein were spared by the oxidation of lactic acid, it is surely likely that the molecules would be broken down into smaller and more soluble moieties, and that these would then be oxidised. But in that case, it is unlikely that they would be oxidised so completely as to leave

absolutely no residual soluble carbon, and if any residual carbon were left, it would be estimated in *OP*, and we should find considerable excess of carbon in *OP*. The same argument applies to the sparing of lipoids. We shall return to this point later, when we discuss experiments which deal directly with the question of nitrogen sparing.

We performed a series of experiments to discover whether the missing lactic acid could have been changed into volatile organic acid. Filtrates were prepared, both by precipitation with mercuric sulphate and also with "colloidal iron," using, on several occasions, 16 g. of brain tissue. In the former case, the sulphuric acid was partly neutralised, to avoid risk of charring when the solution became concentrated; in the latter, the solution was acidified with phosphoric acid. The solutions were then distilled *in vacuo*, and the distillate was caught in standard alkali. The hydrochloric acid, some of which (derived from the chlorides of the Ringer) was always present in the distillate, was estimated with silver nitrate, and was deducted from the total volatile acid.

Small amounts of acetic acid were quantitatively recovered in control experiments; nevertheless, in no experiment was there an excess of volatile acid in *OL* over that found in *OP*; though small amounts were present in both samples. Beyond showing, by treatment with mercuric chloride, that this acid was not formic, we have been unable to identify it.

On another occasion, we repeated the experiment, but distilled into bisulphite, instead of into standard NaOH. Traces only of bisulphite-binding substances were present. We have performed experiments in which brain tissue has been shaken in oxygen in the presence of sulphite and lactate. The oxygen uptake is almost entirely inhibited by the sulphite. On applying the nitroprusside test to the tissue and fluid at the end of the experiment, we were unable to satisfy ourselves that pyruvic acid was present. The amount of bound sulphite was in these cases practically zero, and the same in *OL* and *OP*.

Another possibility which suggested itself, was that synthesis of some organic phosphorus compound was taking place which reduced feebly, or not at all, and was therefore not estimated as carbohydrate. We therefore carried out a series of experiments in which we estimated acid-soluble organic phosphorus in the freshly chopped tissue (initial value) and in the tissue which had been shaken in oxygen, both in the presence and absence of added lactate. These experiments were carried out in bicarbonate buffer with O_2/CO_2 atmosphere, to avoid adding phosphates, and estimations of inorganic phosphate, pyrophosphate, and total acid-soluble organic phosphorus (by incineration with sulphuric acid and "perhydrol") were carried out in the trichloroacetic acid filtrates [Briggs, 1924; Lohmann, 1928].

The results of a series of experiments are shown in Table IX. There is consistently slightly more organic acid-soluble phosphorus in *OL* than in *OP*, but *OL* is consistently lower than the initial value. The "expected synthesis" (reckoned as carbohydrate) in all these experiments is large and cannot be

traced to the synthesis of an acid-soluble organic phosphorus compound. Our total carbon experiments make it highly improbable that some insoluble organic phosphorus compound is being formed.

Table IX. *Determinations of acid-soluble phosphorus fractions.*

All figures are mg. P per 2 g. of tissue.

	Initial				Oxygenated without addition (*OP*)				Oxygenated with lactate (*OL*)			
Date	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.
29. ix. 31	1·585	0·965	0·119	0·501	1·85	1·410	0·090	0·350	1·785	1·270	0·155	0·360
30. ix. 31	1·892	1·200	0·031	0·661	1·768	1·270	0·090	0·408	1·745	1·152	0·072	0·521
3. x. 31	1·612	0·907	0·021	0·684	1·870	1·330	0·060	0·480	1·922	1·320	0·044	0·646
9. x. 31	1·594	0·948	0·036	0·609	1·677	1·337	0·023	0·317	0·184	0·356	0·007	0·437
12. x. 31	2·010	1·390	0·160	0·461	2·005	1·470	0·147	0·391	0·960	1·365	0·145	0·445
,,	,,	,,	,,	,,	2·080	1·626	0·140	0·314	0·820	1·294	0·153	0·373
Average	1·738	1·082	0·073	0·583	1·875	1·407	0·091	0·376	1·841	1·293	0·096	0·464

A further significance attaches to these results, in that the consistently slightly greater figure for acid-soluble organic phosphorus in *OL*, than in *OP*, suggests that a certain small amount of sparing of phosphate ester has taken place. The question of "lipoid sparing" will be discussed elsewhere. Loebel [1925] showed that brain was capable to some extent of oxidising hexose-diphosphate, and our results would be in accordance with this finding.

A series of experiments was undertaken, in which total nitrogen and ammonia were estimated in the mercuric sulphate filtrates from the tissue oxygenated both in the presence and in the absence of lactate. Total nitrogen was estimated by that micro-Kjeldahl method in which alcohol is added and the distillation is assisted by aeration, $N/200$ NaOH being used for titration in the presence of de Wesselow's indicator. Ammonia was determined by Watchorn and Holmes's [1927] modification of Stanford's method [1923].

The results given in Table X show that, except for a single experiment, there is always more total nitrogen in *OP* than *OL*, and invariably more ammonia in the former than in the latter.

Table X. *Total soluble nitrogen and ammonia in samples oxygenated without addition (OP) and in samples oxygenated with added lactate (OL).*

All 4 g. of tissue in O_2 at p_H 7·4 for 3 hrs. at 37°.

	Total N (mg.)			Ammonia-N (mg.)		
Date	*OP*	*OL*	Diff.	*OP*	*OL*	Diff.
17. vii. 31	5·35	4·50	0·85	1·054	0·682	0·372
20. vii. 31	6·55	6·60	−0·05	1·256	0·657	0·599
24. vii. 31	6·05	5·29	0·76	1·247	0·772	0·475
30. vii. 31	4·01	3·31	0·70	1·130	0·768	0·362

Loebel [1925] has already shown that less ammonia is formed by brain tissue in oxygen in the presence of glucose or of lactate, and his results are confirmed, with regard to the latter substance, by the evidence which we

present here. The interpretation of these experiments is not at present easy. Clearly, in view of the figures for total carbon, nitrogen which would otherwise be insoluble in mercuric sulphate (*e.g.* protein- or lipoid-nitrogen) cannot have been built with the lactic acid into some nitrogenous compound; but our results would not preclude the possibility of nitrogen already present in some soluble form being transferred to a new compound derived from the lactic acid.

The fact that there is more ammonia in *OP* than in *OL* suggests strongly that the presence of the lactate has inhibited deamination. Pohle [1929] has demonstrated the presence of adenylic acid in brain tissue, and Holmes and Holmes [1925] had earlier stated that pentose was present in brain. At the same time, B. E. Holmes had isolated hypoxanthine, in the form of hypoxanthine silver nitrate, from brain tissue, but as it was subsequently found that Thudichum [1884] had made the same observation at a much earlier date, and as the significance of adenylic acid was not then realised, no mention of this finding was made. In view of this, and of the importance of the deamination of adenine in muscle, it seems very likely that the ammonia is derived, at least partly, from adenine, and that this process is partly inhibited in the presence of lactate. In this case, it is possible that the breakdown of pyrophosphate might also be inhibited. We have, however, found no evidence that this occurs. Table VIII shows that very little pyrophosphate is to be found in the brain under the conditions of our experiments, and that there is no suggestion that its amount is altered by the presence of lactate.

The alternative view—the one which is supported by the change observed in total nitrogen—is that the effect of the lactate is actually to spare protein or lipoid, and that part at least of the N in *OP* represents the deamination of protein or lipoid, or of both; but if such is the case, it must be remembered that the total carbon figures show that the remainder of the molecule must have undergone almost complete combustion.

Loebel [1925] suggests that, if protein is burned, 4–5 molecules of oxygen should be used for every molecule of nitrogen split off. Table IX shows (neglecting the second experiment) that about 0·77 mg. of ammonia-N was apparently "spared" by the lactate. This represents $0{\cdot}77/14 = 0{\cdot}055$ millimoles of ammonia-N, and should, in turn, represent about $0{\cdot}055 \times 4{\cdot}5$ or 0·246 millimoles of oxygen. This oxygen would completely oxidise $\frac{0{\cdot}246}{3} = 0{\cdot}082$ millimoles, or 7·38 mg. of lactic acid.

Lusk [1928] from Loewy's data, states that, in the animal body, 100 g. of (meat) protein would require 138·18 g. of O, and that 8·49 g. of O would be required per g. of urinary N. Thus, 1 mg. of N should correspond to $\frac{138{\cdot}18}{8{\cdot}49} = 16{\cdot}28$ mg. of O and 0·77 mg. would correspond to $\frac{138{\cdot}18}{8{\cdot}49} \times 0{\cdot}77 = 12{\cdot}53$ mg. of O, *i.e.* to $\frac{12{\cdot}53}{32} = 0{\cdot}393$ millimoles of O. This would account for the complete oxidation of $\frac{0{\cdot}393}{3} = 0{\cdot}131$ millimoles, or 11·79 mg. of lactic acid.

Now the average figure which we obtain for the amount of lactic acid

which disappears and is not accounted for by the extra oxygen, is, for 4 g. of brain, 14·68 mg. (mean of 7 experiments in which total carbon is estimated). It will be seen therefore that only about three-fifths of the lactic acid could, on the basis of these figures, have been used for "protein sparing."

If, on the other hand, lipoid (*e.g.* lecithin), instead of protein were spared, and if we assume that the molecular weight of lecithin is 773, then 1 mg. of nitrogen would correspond to 221 mg. of lactic acid. It will be seen, however, from Table IX that the total acid-soluble phosphorus is practically identical in amount both in *OP* and *OL*. It is difficult to picture any manner in which lecithin could be broken up without giving rise to soluble phosphorus compounds, and it seems, therefore, fair to discount the possibility of lipoid sparing having occurred. Table IX shows also that there is only a small liberation of soluble phosphorus in either *OL* or *OP*, the increase being only 0·137 mg. of P in the one case and 0·013 mg. in the other. This is in conformity with the relatively high R.Q. (0·9 and over) which is found even in the absence of lactate. It also falls into line with the experiments of King [1931] who finds that brain tissue does not break down lecithin at all readily.

Table XI.

Date	Lactic acid in *OL* after 1½ hrs. shaking in O_2 mg.	Lactic acid in *OL* after 1½ hrs. in O_2 followed by 1½ hrs. in N_2 mg.	Lactic acid after 1½ hrs. in O_2 followed by addition of glucose, and 1½ hrs. in N_2 mg.	Lactic acid in *OP*, after shaking 1½ hrs. in O_2, followed by glucose and 1½ hrs. in N_2 mg.
4. viii. 31	5·75	5·75	—	—
6. viii. 31	6·40	6·50	14·6	10·05

In the course of experiments still in progress [unpublished], Sherif and Holmes have observed (in confirmation of previous workers McGinty and Gesell [1925], Kinnersley and Peters [1929], and Jungmann and Kimmelstiel [1929]) that if brain tissue is very rapidly cooled in liquid air (in these experiments mice were used), a comparatively low value for lactic acid is obtained, which rises on incubation at 40°. Simultaneous estimations of total carbohydrate show a fall in the latter, coincident in time with the rise in lactic acid, but sometimes less in amount (compare work of Kerly [1931] on muscle). In other words, lactic acid appears from some source other than substances estimated as carbohydrate. It seemed possible that this unindentified lactic acid precursor might, in fact, be the substance into which lactic acid was changed in our own experiments. To test this, samples of brain with added lactate were shaken for 1½ hours in oxygen. One sample was then analysed for lactic acid. The Barcroft apparatus containing another similar sample was then filled with nitrogen, and shaken for a further period of 1½ hours. Analysis showed that this had exactly the same lactic acid content as the sample taken for estimation after 1½ hours in oxygen—no reappearance of lactic acid had taken place under anaerobic conditions. Control experiments showed that,

after 1½ hours' shaking in oxygen, the tissue in nitrogen could still freely form lactic acid from glucose. These results are illustrated by Table XI.

Evidently the lactic acid is not changed into the unknown precursor, which exists, apparently, in the intact brain.

Discussion.

From the experimental evidence before us, we must conclude either that the larger part of the lactic acid which disappears when brain tissue is shaken with lactate in the presence of oxygen is transformed into some substance which has so far eluded identification, or else that all the lactic acid which disappears is in reality oxidised, but in the process "spares" some other substance or substances from oxidation.

The points in favour of the latter explanation are, first, its inherent plausibility. The principle of the "sparing" of one substrate by another in the animal body is well established, and there is no obvious reason why it should not apply to individual isolated organs. Indeed, the occurrence of nitrogen sparing by sugars in the growing kidney has been demonstrated by Watchorn and B. E. Holmes [1927, 1931]. From our own experiments we know that there is actually more soluble nitrogen in brain tissue that has been shaken in oxygen in the absence of lactate than its presence.

Brain tissue certainly contains oxidisable substances other than lactic acid—Thudichum [1884] demonstrated the presence of succinate, and succinate takes up oxygen even more freely than lactate—and probably there are others. The fact that 95 % of the carbon corresponding to the missing lactic acid can be found as soluble carbon is no bar to this explanation, if the line be taken that some soluble, carbon-containing substrate is spared; there is no means of identifying the carbon as derived especially from lactic acid. But it does bear on the question of protein or lipoid sparing, in the sense that it means that, if such sparing occurs, then practically the whole protein or lipoid molecule must be spared. We picture, after all, that hydrolysis must precede oxidation; and if this is so, all the hydrolysed fragments must be completely oxidised—if any were left unoxidised, they would upset the carbon balance. But this complete oxidation seems improbable. Against this explanation too, is the fact that, in tissue depleted of substrate by various methods, more lactic acid disappears than can be accounted for by the oxygen uptake (Table V). Here an explanation on the lines of a "sparing" effect is clearly impossible. We are thrown back on the assumption that some substance is present which, so far, we have failed to identify.

Still further evidence against the "sparing" hypothesis is afforded by a consideration of Figs. 2 and 3 and of Table IV, which shows the effect of varying concentrations of added lactate. We may assume that we have initially present in the tissue unknown substrates, which we may denote by X, and a certain amount of lactic acid l. When shaken in oxygen, suppose these amounts

become X_0 and l_0 (*i.e.* the value given as *OP* in the Tables). In the case of *OL* an amount L of lactic acid was added to the tissue, giving $(X + l + L)$ initially. After shaking in oxygen, suppose the amounts become $x_0 + l_0 + L_0$.

If now $X_0 = x_0$, then the differences in the amounts of oxygen taken up in the two cases should be due to the added lactic acid. If, however, X_0 is not equal to x_0 and is less than x_0, competitive inhibition or "sparing" has taken place. If this inhibition is of a competitive nature [Haldane, 1930], then an increase of L should cause a diminution in the amount of X oxidised, *i.e.* x_0 should be greater than X_0. In effect more of the oxygen taken up will have been used in the oxidation of the lactic acid than is shown by the difference of the two oxygen uptakes, *i.e.* more lactic acid is disappearing but the amount of "extra oxygen" remains the same. Hence the Meyerhof quotient should increase as we increase the concentration of the added lactate. On the other hand, assuming that, in actual fact, total oxidation of lactic acid takes place as the R.Q. suggests, the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

should approach unity or fall as the lactic concentration is increased. It should be noted that 2 g. of the chopped tissue would contain about 2·7 mg. of lactic acid, and that this amount is almost maximal, *i.e.* aerobic glycolysis does not take place (as it does in muscle) except to a very small extent, owing to the absence of precursor in the tissue [Holmes and Holmes, 1925]; Ashford

Table XII. *Ratio of* $\frac{\textit{Oxygen equivalent of total lactic acid removed}}{\textit{Total oxygen uptake}}$ *at different initial concentrations of added lactate.*

Approx. conc. Li lactate %	Initial lactic acid g.	Final lactic acid g.	Lactic acid removed g.	O_2 equiv. of lactic acid removed mm.³	Total O_2 uptake mm.³	Ratio
0·025	3·7	0·56	3·14	2,350	4710	0·5
0·05	4·7	0·68	4·02	3,000	4875	0·615
0·1	6·9	1·6	5·31	4,450	5000	0·89
0·2	11·97	2·885	9·1	6,750	6450	1·05
0·3	17·0	4·65	12·35	9,200	6700	1·37
0·4	18·4	7·31	11·1	8,300	6480	1·28
0·5	22·4	10·32	11·1	8,260	6300	1·31
0·6	26·7	13·2	13·5	10,000	7110	1·40
0·8	34·2	20·1	14·1	10,500	7000	1·44

and Holmes, 1929]. At the lowest concentrations employed therefore, the amount of added lactic acid is small, or comparable with the amount already in the tissue. The findings have already been noted, *viz.* that at the lowest concentrations the Meyerhof quotient is smaller but that between 0·3 % and 0·8 % there is little change. This makes it unlikely that competition between substrates is taking place, or in other words, probably no "sparing" occurs. Further evidence of this is given in Table XII. It will be seen that the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

is less than unity at low concentrations, but afterwards increases above unity, whereas if the suggestion made above were correct, it should decrease with increased concentration of lactate.

Summary.

1. Experiments are described in which it is shown that a Meyerhof quotient can be obtained if brain tissue is shaken with lactate in the presence of a bicarbonate buffer, in an O_2/CO_2 atmosphere. A technique is described by which the use of manometric methods is avoided. In spite of the existence of the "quotient," no carbohydrate synthesis occurs.

2. The O_2 uptake is greater in the presence of a CO_2-bicarbonate buffer than in the presence of phosphate and increases with increased oxygen tension in both cases.

3. There is little difference between the oxygen uptake of intact and chopped frog's brain, measured in O_2 at room temperature, in the presence of phosphate buffer.

4. The respiratory quotients, both of the brain tissue alone and of the "extra oxygen," are close to unity, even in the case of animals rendered hypoglycaemic by insulin injection.

5. With brain tissue, glucose, lactate, succinate and glycerophosphate all act as hydrogen donators to methylene blue. Glycerol and dihydroxyacetone are without effect on the reduction time; formate, acetate and mandelate prolong it.

6. Estimations of total carbon in protein-free filtrates of tissue shaken with and without lactate indicate that the "extra oxygen" gives a true measure of the lactic acid oxidised.

7. There is no evidence of formation of volatile acid, or of any substance capable of binding bisulphite, from lactic acid in the presence of brain tissue.

8. Inorganic, pyro-, and acid-soluble organic phosphorus have been estimated. There is evidence of a small increase both in inorganic and in total phosphorus, at the expense of organic phosphorus, both in the presence and in the absence of lactate; but there is no evidence of the synthesis of any phosphorus compound from the fraction of the lactic acid which disappears and is not accounted for by oxidation.

9. There is more non-protein nitrogen, and more ammonia, in filtrates from brain tissues which have been aerated in the absence of lactic acid, than in its presence.

10. The possibility of accounting for the disappearance of lactate on the lines of a "sparing" effect on some other substrate is discussed, and it is shown that such an explanation is probably not the correct one.

Our thanks are due to Sir F. G. Hopkins, for his interest in this work.

REFERENCES.

Ashford and Holmes (1929). *Biochem. J.* **23**, 748.
Briggs (1924). *J. Biol. Chem.* **59**, 255.
Case and Cook (1931). *Biochem. J.* **25**, 1319.
Dixon and Elliott (1929). *Biochem. J.* **23**, 812.
Haldane, J. S. (1920). Methods of air analysis. (Griffin.)
Haldane, J. B. S. (1930). Enzymes, p. 7. (Longmans, London.)
Holmes (1930). *Biochem. J.* **24**, 914.
—— and Ashford (1930). *Biochem. J.* **24**, 1119.
—— and Holmes (1925). *Biochem. J.* **19**, 496.
Jungmann and Kimmelstiel (1929). *Biochem. Z.* **212**, 347.
Kerly (1931). *Biochem. J.* **25**, 671.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
King (1931). *Biochem. J.* **25**, 799.
Loebel (1925). *Biochem. Z.* **161**, 219.
Lohmann (1928). *Biochem. Z.* **202**, 466.
Lusk (1928). The science of nutrition. (Saunders, London.)
Meyerhof (1919). *Pflüger's Arch.* **175**, 20.
McGinty and Gesell (1925). *Amer. J. Physiol.* **75**, 70.
Neuberg and Kobel (1930). *Ber. deutsch. chem. Ges.* **63**, 1039.
Pohle (1929). *Z. physiol. Chem.* **185**, 281.
Quastel and Wheatley (1931). *Biochem. J.* **25**, 117.
Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**.
Stanford (1923). *Biochem. J.* **17**, 847.
Thudichum (1884). The chemical constitution of the brain. (Baillière, Tindall and Cox, London.)
Toennissen and Brinkmann (1930). *Z. physiol. Chem.* **187**, 137.
Warburg (1926). Ueber die Stoffwechsel der Tumoren, p. 198. (Springer, Berlin.)
Watchorn and B. E. Holmes (1927). *Biochem. J.* **21**, 1391.
—— —— (1931). *Biochem. J.* **25**, 843.

CCXXII. THE DISTRIBUTION OF THE VITAMIN B COMPLEX.

III. FRUITS.

By MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

(*Received October 31st, 1931.*)

Most of the previous work on the distribution of vitamin B in fruits was carried out before the differentiation of the complex into the antineuritic vitamin B_1 and the antidermatitis vitamin B_2, and as a rule the results of the older experiments give only rough indications of the presence of both constituents of the complex. These have been summarised by Sherman and Smith [1931].

In the work described in the present paper, apple, banana, orange and tomato, the fruits in most common use in this country, were tested for their vitamin B_1 and vitamin B_2 content respectively. Vitamin B_4 [Reader, 1929; 1930, 1, 2] has not been tested for independently. As it was not being supplied in the basal diet used for the vitamin B_1 tests, these indicated the content of vitamin B_1 and B_4 combined.

This work is a continuation of that on the vitamin B_1 and B_2 content of foodstuffs previously carried out in this laboratory [Aykroyd and Roscoe, 1929; Aykroyd, 1930; Chick and Copping, 1930; Roscoe, 1930, 1931]. A comparison is given in this paper of all the results hitherto obtained.

Methods.

The methods employed were those described in the previous papers [Roscoe, 1930, 1931], in which the growth of young rats is used as criterion.

In the vitamin B_1 estimations the diets contained unpurified caseinogen and the vitamin B_2 was supplied as autoclaved yeast extract (F5A). For the vitamin B_2 estimations purified caseinogen was used and vitamin B_1 was provided as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925].

The fruits were prepared by peeling, coring or removing the pips, and then mincing. The water content of the pulps so obtained was reduced by exposing to the air in thin layers at 37°. The fruits were bought from time to time throughout the experiments. No particular variety was adhered to.

The reduced pulps were incorporated in the diets in varying percentages (reckoning from the dry weights), but the rats were only allowed to consume

[1] Beit Memorial Fellow.

enough of these diets to provide a given amount of the fruit each day, the remainder of their requirements being satisfied with a vitamin-free diet.

THE VITAMIN B_1 CONTENT OF CERTAIN FRUITS (Table I).

Table I. *Vitamin B_1 content of certain fruits as demonstrated by weight increase of rats during 5 weeks on diets containing various percentages of the fruit pulps.*

(Vitamin B_2 supplied as autoclaved yeast extract (F5A).)

Material tested	Amount eaten daily: Wet weight g.	Amount eaten daily: Dry weight g.	No. of rats	Increase in weight in 5 weeks (averages, g.)
Orange	2·5	0·25	4	50
	5·0	0·5	1	85
Tomato	6·25	0·5	4	38
	12·5	1·0	4	82
Banana	2·3	0·5	2	No growth
	4·6	1·0	4	51
Apple	5·5	1·0	4	32
	11·0	2·0	4	66

The amounts referred to in the following paragraph are the dry weights consumed daily.

Orange proved a rich source of the antineuritic vitamin; rats eating as little as 0·25 g. gained 50 g. in weight in 5 weeks, those eating 0·5 g., 85 g. *Tomato* was about half as good as orange; rats receiving 0·5 g. gained 38 g., those with 1·0 g., 82 g. *Banana* was one-quarter as rich as orange; with 1·0 g. the increase in weight was only 51 g. *Apple* was even poorer; with as much as 2·0 g. the increase in weight in 5 weeks was only 66 g.

In Table II the results obtained with these fruits, together with the values for the other foodstuffs previously published, are tabulated in the order of their vitamin B_1 potency. The amounts shown are the dry weights needed by

Table II. *The vitamin B_1 content of various foodstuffs (dry weights).*

Material	Daily dose needed to promote increase in wt. of 50–60 g. in 5 weeks g. dry wt.		Potency (yeast=100)
Yeast (brewer's)	0·05	<0·25	100
Wheat germ	0·1		50
Liver (ox)	<0·5	0·25–0·5	10–20
Watercress	<0·3		
Lettuce	0·3		
Orange	0·25		
Etiolated cabbage	0·3 –0·5		
Carrot	0·25–0·5		
Spinach	<0·5		
Green cabbage	<0·95	0·5–1·0	5–10
Turnip	0·5–1·0		
Tomato	0·5–1·0		
Egg-yolk	0·5–1·0		
Onion	0·5–1·0		
Meat (ox muscle)	1·0–1·5	1·0–2·0	2·5–5·0
Banana	1·0		
Potato	1·0–2·0		
Apple	1·0–2·0		

young rats, when given as sole source of vitamin B_1, to produce an increase in weight of 50–60 g. in 5 weeks. These values have been used to calculate the vitamin B_1 potency in terms of that of yeast. Yeast being taken as 100, the materials are placed in groups with potencies of over 20, 10–20, 5–10 and 2·5–5, respectively.

It will be seen that the orange is as good a source of vitamin B_1 as any of the substances tested, except yeast and wheat germ. Its potency lies between 10 and 20 and is equal to those of the green leaf vegetables, liver and carrot. Tomato has a potency of between 5 and 10, equal to those of turnip, egg-yolk and onion. Banana and apple have potencies between 2·5 and 5, equal to those of meat and potato.

The vitamin B_2 content of certain fruits (Table III).

Orange, *tomato* and *banana* all proved to be equal sources of this vitamin. In each case rats receiving 1·0 g. daily increased in weight by about 30 g. in 5 weeks and those receiving 2·0 g. by about 60–70 g. *Apple* was again the

Table III. *The vitamin B_2 content of certain fruits as demonstrated by weight increase of rats during 5 weeks on diets containing various percentages of the fruit pulps.*

(Vitamin B_1 supplied as antineuritic yeast concentrate (YE).)

Material tested	Amount eaten daily: Wet weight g.	Amount eaten daily: Dry weight g.	No. of rats	Increase in weight in 5 weeks (averages, g.)
Orange	10·0	1·0	4	29
	20·0	2·0	5	69
Tomato	12·5	1·0	2	No growth
	25·0	2·0	4	67
Banana	4·6	1·0	4	35
	9·2	2·0	4	62
	18·4	4·0	3	102
Apple	5·5	1·0	2	34
	11·0	2·0	4	47
	22·0	4·0	4	66

poorest of the fruits tested; rats receiving 1·0 g. increased in weight 34 g. in 5 weeks but 4·0 g. were needed to produce 66 g. increase.

In Table IV these fruits are placed in order of their vitamin B_2 contents, together with the other foodstuffs previously tested. The amounts needed to promote an increase in weight of 50–60 g. in 5 weeks are given and from these have been calculated their potencies when yeast is taken as 100.

The fruits tested do not stand very high as regards their vitamin B_2 content; it was lower than the vitamin B_1 content. Orange, tomato and banana are equal to wheat germ and onion, poorer than the green leaf vegetables, root vegetables, meat and milk, but richer than the cereals, pulses or potatoes. Apple has a content equal to that of the cereals.

In Table V are shown the vitamin B_1 and vitamin B_2 potencies, respectively, of the various foodstuffs, when the fresh weights needed to promote

Table IV. *The vitamin B_2 content of various foodstuffs (dry weights).*

Material	Daily dose needed to promote increase in wt. of 50–60 g. in 5 weeks g. dry wt.		Potency (yeast = 100)
Yeast (brewer's)	0·1	< 0·25	100
Liver (ox)	0·12		80
Egg-white	0·3–0·6	0·25–0·5	20–40
Watercress	0·4		
Green cabbage	0·4		
Spinach	0·4		
Milk (cow's)	0·7	0·5–1·0	10–20
Meat (ox muscle)	0·5–0·75		
Lettuce	0·6		
Etiolated cabbage	0·4–0·8		
Carrot	0·5–1·0		
Turnip	1·0		
Egg-yolk	1·0		
Wheat germ	1·0–2·0	1·0–2·0	5–10
Onion	1·6		
Orange	1·0–2·0		
Banana	1·0–2·0		
Tomato	<2·0		
Peas (dried)	2·0–3·0	2·0–4·0	2·5–5
Wheat (whole)	2·0–3·0		
Apple	2·0–4·0		
Potato	2·0–4·0		
Maize (whole, white)	2·0–3·5		
Millet	>4·0	>4·0	<2·5
Maize (whole, yellow)	>4·0		
Rice	>4·0		

Table V. *The vitamin B_1 and B_2 potencies of various fresh foodstuffs.*

The potencies have been calculated by comparing the amounts of the fresh materials needed to promote 50–60 g. growth in young rats during 5 weeks, with the amount of fresh yeast needed (*i.e.* as source of vitamin B_1, 0·05 g., as source of vitamin B_2, 0·1 g.).

Potency	Vitamin B_1	Vitamin B_2
More than 100	Wheat germ	Liver (ox)
100	Yeast (brewer's)	Yeast (brewer's)
20–100	Liver (ox)	Wheat germ
	Egg-yolk	Egg-yolk
10–20		Meat (ox muscle)
		Egg-white
		Watercress
		Peas (dried)
		Wheat (whole)
		Spinach
		Maize (whole, white)
		Green cabbage
5–10	Meat (ox muscle)	Milk
	Watercress	Lettuce
	Orange	Etiolated cabbage
	Lettuce	Carrot
	Spinach	Banana
	Etiolated cabbage	
	Carrot	
	Banana	
2·5–5	Potato	Potato
	Green cabbage	Apple
	Tomato	Orange
	Turnip	Tomato
	Apple	Turnip
Less than 2·5	Onion	Onion

50–60 g. growth in 5 weeks are compared with the amount of fresh yeast needed.

It is noticeable that with a few exceptions the materials tested do not vary widely in their content of vitamins B_1 and B_2 respectively. Yeast, wheat germ, liver and egg-yolk have potencies of both vitamins greater than 20, but all the remaining foods lie between 2·5 and 20, with the exception of onion which is lower.

A general similarity is also exhibited in the distribution among the foodstuffs of the two vitamins, the materials when placed in order of value forming two very similar series (see Table V). There are however marked exceptions. Liver and meat have a considerably higher proportion of vitamin B_2 than of B_1 (vitamin B_1 potency/vitamin B_2 potency = 1/4), and the green leaf vegetables, egg-yolk, banana and turnip a slightly higher proportion (1/2), that is, these take higher places in the vitamin B_2 series than in the vitamin B_1 series. On the other hand, wheat germ has a much higher proportion of vitamin B_1 than of B_2 (8/1), and orange and the whole cereals a slightly higher (2/1); these, therefore, take higher places in the vitamin B_1 series.

Egg-white is unique in that it is the only known natural source of vitamin B_2 without vitamin B_1. No natural source of vitamin B_1 without vitamin B_2 is known.

These results may be compared with those of previous investigators. Apples have always been found to be poor sources of "vitamin B" [Weatherby and Waterman, 1928]. Oranges were here found to be richer in the antineuritic vitamin, than had been thought, about 4 cc. of juice, not 10 cc. [Sherman, 1926], being sufficient for normal growth when vitamin B_2 was being adequately supplied; 20 cc., not 10 cc. [Willimott, 1928], were needed, however when the juice was the sole source of vitamin B_2. Tomatoes, as previously shown [House, Nelson and Haber, 1929], were here found to be rather poorer than oranges in vitamin B_1. Bananas were tested for their vitamin B_1 and B_2 contents separately by Eddy [1927]. The present assays do not agree with his. This may be due to the fact that Eddy based his estimations on weight maintenance for both vitamins, whereas the weight of rats can be maintained without vitamin B_2 if vitamin B_1 is supplied. On this basis the vitamin B_2 potency was found by him to be considerably higher than the vitamin B_1 potency, whereas in this work it was lower. In Eddy's work also 6 g. of fresh banana when given as source of vitamin B_2 were found to support nearly normal growth, here more than 17 g. were needed.

Summary.

1. The vitamin B_1 content of orange was the highest of the fruits tested, being one-fifth that of yeast (dry weights) and equal to that of the green leaf vegetables or liver. The value of tomato was rather less than one-tenth that of yeast, banana one-twentieth and apple still lower.

2. The vitamin B_2 content of the fruits tested was lower. The value of

orange, tomato and banana was rather less than one-tenth and of apple about one-twentieth that of yeast (dry weights).

3. The vitamin B_1 and B_2 potencies of all the foodstuffs tested in this laboratory have been calculated taking yeast as standard. These are tabulated in order of magnitude, firstly comparing dry weights and secondly fresh weights.

4. A large number of the foodstuffs tested had the same proportion of vitamin B_1 to vitamin B_2 as had yeast. Liver, meat, the green leaf vegetables and egg-yolk were richer in vitamin B_2 than in vitamin B_1, while the reverse was true of wheat germ, orange and the whole cereals.

My thanks are due to Dr H. Chick for advice and criticism throughout the course of this work, and to Miss D. Graetz for help with the experimental animals.

REFERENCES.

Aykroyd (1930). *Biochem. J.* **24**, 1479.
—— and Roscoe (1929). *Biochem. J.* **23**, 483.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Eddy (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 125.
House, Nelson and Haber (1929). *J. Biol. Chem.* **81**, 495.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
Peters (1924). *Biochem. J.* **18**, 858.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Roscoe (1930). *Biochem. J.* **24**, 1754.
—— (1931). *Biochem. J.* **25**, 1205.
Sherman (1926). Chemistry of food and nutrition, 3rd edition, p. 406. (New York.)
—— and Smith (1931). The vitamins, 2nd edition. (New York.)
Weatherby and Waterman (1928). *J. Ind. Eng. Chem.* **20**, 968.
Willimott (1928). *Biochem. J.* **22**, 67.

CCXXIII. THE EFFECTS OF COPROPHAGY IN RATS DEPRIVED OF THE VITAMIN B COMPLEX.

By MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

(*Received October 31st, 1931.*)

Introduction.

The presence of "vitamin B" in the excreta of birds and mammals has long been established. The antineuritic vitamin was found by Cooper [1914] in alcoholic extracts of faeces from hens and rabbits receiving normal diets, and Portier and Randoin [1920] found it in the faeces, but not in the urine, of rabbits which had been kept on diets deficient in vitamin B. Its presence was attributed by these authors either to unabsorbed residues from the food or to synthesis by bacteria in the intestines.

Muckenfuss [1918], Gaglio [1919] and Van der Walle [1922] all found the antineuritic vitamin in adsorbates from the urine of animals fed on normal diets, but it was reduced in starving animals and absent after 3 weeks' deprivation of "vitamin B."

Later it was observed that animals on diets completely or partially deficient in "vitamin B" frequently consumed their own faeces, the result being, when suboptimal doses of the vitamin were being supplied, a greatly improved rate of growth. Thus Steenbock, Sell and Nelson [1923] observed that such rats grew normally with half as much "vitamin B" in the diet as was needed by the non-coprophagous rats. They considered that an additional supply of "vitamin B" was being obtained from the unabsorbed residue of the food, since in absence of "vitamin B" from the diet coprophagy did not prolong life.

Dutcher and Francis [1923], however, kept coprophagous rats on diets completely deficient in "vitamin B" for 5–7 months. They considered this prolongation of life to be due to the re-ingestion of excreted stores of "vitamin B," an explanation supported by the failure of rats to benefit by coprophagy if it was not started till after the first few weeks of vitamin depletion, when the stores of the vitamin were exhausted [Steenbock, Sell and Jones, 1923].

The limited capacity of animals for storing "vitamin B" made it improbable that their stores, even when wastage through the faeces was prevented, could be sufficient to protect against symptoms of "vitamin B" deficiency during the long periods of observation. It was suggested that only minute

[1] Beit Memorial Fellow.

quantities of the vitamin were used at a time, or that none was actually destroyed, the action being catalytic, but against these theories there is the certainty of loss in the portion of the faeces which is not consumed, and the probability of loss in the urine. The theory was therefore advanced that "vitamin B" was synthesised by bacteria in the alimentary canal of coprophagous rats and evacuated with the faeces, from which it was assimilated. This was the more likely as a number of bacteria have been shown to synthesise the vitamin B complex even when grown on synthetic media free of any traces of the vitamin [Schieblich, 1929, 1930, 1, 2].

In support of this theory Heller, McElroy and Garlock [1925] found a marked increase in spore-bearing organisms, which were able to synthesise "vitamin B," in the faeces of coprophagous rats. These had completely disappeared, however, after 3 weeks.

Bechdel *et al.* [1928] found that cows could be reared to maturity on "vitamin B"-deficient diets. Their rumens were shown to contain the vitamin, as the result, it was thought, of synthesis by a small unknown bacterium which was isolated. Coprophagy did not seem to be necessary in order that the vitamin complex should be obtained from the bacteria or their products.

In none of these researches was the vitamin B complex differentiated into its components. When rats were used the experiments dealt therefore with the whole complex; when the tests were based on the prevention or cure of polyneuritis in birds the vitamin B_1 was alone being considered.

Kennedy and Palmer [1928] showed that both vitamins B_1 and B_2 were supplied in the faeces and considered that a third factor was also present supplementing the "vitamin B"-deficient diet; this appears, however, to have been due to an inadequate supply of vitamin B_2.

Scheunert, Schieblich and Rodenkirchen [1929] studied the effect of coprophagy in rats receiving diets containing raw starch, but the greater number of their rats became refected [Fridericia *et al.*, 1927; Roscoe, 1927]. These workers did not apparently realise the difference between their results and those obtained by other workers on coprophagy in whose experiments refection had been prevented by using dextrin in the diet instead of starch [Roscoe, 1927].

Subsidiary reasons, which have been put forward to account for the improved condition of coprophagous animals, are (i) that the faeces act as roughage which is thought to prolong the existence of "vitamin B"-deficient animals [Heller, McElroy and Garlock, 1925], and (ii) that these rats do not suffer from the same disabling discomfort as those in which coprophagy is prevented by the use of cages with open wire floors [McCollum, Simmonds and Becker, 1925].

In this paper experiments are described involving an accurate investigation of coprophagy in rats deprived of the entire vitamin B complex, of vitamin B_1 alone or of vitamin B_2 alone. An attempt was also made to determine the origin of the B-vitamins which these rats acquired through eating their faeces, and to see whether this was similar to that of the vitamin B obtained by refected rats.

Methods.

The rats were kept in the cages used in this laboratory for all work on the B-vitamins. These are made of perforated zinc with floors of galvanised wire netting of $\frac{1}{2}$ inch mesh. The floors of the cages, through which the faeces fall freely, are raised 2″ above the peat bedding. In some of the experiments the rats were housed in glass jars 6″ in diameter, on raised floors made from the same wire netting. Both the coprophagous rats and their controls were kept in these cages; no results could therefore be attributed to differing degrees of comfort enjoyed by the rats.

The peat was covered with a fine wire gauze which allowed the passage of the urine but not of the faeces, from this floor the faeces were collected daily. They were dried overnight at 37°, ground, in some cases weighed, and fed to the rats on the following day. In this form they were more readily consumed than when fresh.

The rats were fed on a basal diet complete except for B-vitamins. Vitamins A and D were supplied as a daily dose of cod-liver oil. The diet consisted of caseinogen 20 parts, rice starch 60, salt mixture 5, cotton-seed or arachis oil 15 parts. This diet was usually steamed for 3–4 hours before using so that the starch was rendered completely soluble.

When vitamins B_1 and B_2 were given these were provided as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925] and as an autoclaved yeast extract (F5A) [Roscoe, 1930], respectively.

No distinction has been made in this work between vitamins B_1 and B_4 [Reader, 1929, 1930, 1, 2] but it was known that the yeast concentrate used as source of vitamin B_1 having been made with acid alcohol contained vitamin B_4 [Reader, 1929]. The yeast extract used as source of vitamin B_2, having been autoclaved for 5 hours, would not contain any vitamin B_4. The rats were, therefore, either deficient in vitamins B_1 and B_4, or deficient only in vitamin B_2.

Young rats were weaned at the age of 3 weeks and received the basal diet and cod-liver oil as soon as they weighed 40 g. Litters 1,000, 2,000 and 4,000 then received their supplementary doses of faeces. Litters 45,000, 51,000 and 52,000 all received their doses of faeces and of vitamins B_1 or B_2 after 1 week on the basal diet alone.

Experimental.

Exp. 1. Growth and prolongation of life in rats deprived of the entire vitamin B complex.

Litters as in Table I.

In Table I are collected the data relating to coprophagous rats and their non-coprophagous litter-mates on a diet deficient in the whole vitamin B complex.

The results with rats receiving an uncooked diet will be considered under Exp. 5.

The coprophagous rats on the cooked diets all remained alive for much longer than their controls. Three developed neuritis after 9½, 9½ and 13 weeks respectively and four after 21, 22, 35 and 36 weeks. The rest were in good health when the experiments were terminated after 8 weeks (3 rats), 19 weeks (2 rats) and 9, 10, 15 and 16½ weeks respectively.

For the greater part of the period of observation the rats did not grow

Table I. *Life histories of coprophagous rats and their non-coprophagous litter-mates on diets deficient in the entire vitamin B complex.*

(In brackets are given the average weekly weight increase.)

Rat No.	Coprophagous	Rat No.	Non-coprophagous
Uncooked diet:			
1,005 ♂	Lost weight for 4 weeks. Died	1,009 ♂	Became refected after 1 week
1,004 ♂	Became refected after 1 week	1,010 ♀	Lost weight for 3 weeks. Dosed
1,006 ♂	,, ,, 2 weeks		
2,008 ♂	Lost weight for 4 weeks. Died	2,006 ♂	Lost weight for 3 weeks. Died
2,009 ♀	,, 3 ,, Dosed	2,007 ♀	,, 4 ,, ,,
		2,010 ♂	,, 3 ,, Dosed
Cooked diets:			
1,001 ♂	Slow growth for 8 weeks (1 g.). Experiment stopped	1,007 ♂	Neuritis after 6 weeks
1,002 ♂	,, 8 ,, (3 g.). ,,	1,008 ♂	Lost weight for 4 weeks. Dosed
1,003 ♀	,, 8 ,, (2 g.). ,,		
2,001 ♂	*Grew (5 g.) for 16 weeks with one break of 4 weeks.* Lost weight. Neuritis after 22 weeks	2,002 ♀	Lost weight for 3 weeks. Died
2,011 ♂	Slow growth for 8 weeks (1 g.). *Grew for 9 weeks (6 g.).* Stationary then lost weight. Neuritis after 21 weeks	2,003 ♂	,, 5 ,, Dosed
2,004 ♀	*Grew (5·5 g.) for 16 weeks with one break of 3 weeks.* Stationary then lost weight. Neuritis after 35 weeks		
2,005 ♂	4 weeks (0 g.). *Grew (7 g.) for 13 weeks with one break of 3 weeks.* Stationary then lost weight. Neuritis after 36 weeks.		
4,004 ♀	Weight stationary for 9 weeks. Neuritis after 9½ weeks	4,001 ♂	Lost weight for 5 weeks. Died
*4,006 ♂	,, ,, 9 ,, ,, 9½ ,,		
4,002 ♂	Grew for 8 weeks (3 g.). Stationary for 5 weeks. Neuritis after 13 weeks		
*4,005 ♂	Grew for 11 weeks (2 g.). *Then for 8 weeks (6 g.).* Experiment stopped		
4,003 ♀	,, 11 ,, (1·5 g.). *Then for 8 weeks (8 g.).* ,,		
*4,007 ♀	,, 11 ,, (2 g.). *Then for 5½ weeks (6 g.)* ,,		
51,384 ♂	,, 10 ,, (1 g.) Experiment stopped	51,383 ♂	Lost weight for 5 weeks. Neuritis
52,390 ♂	,, 6 ,, (2 g.). Lost weight for 3 weeks. Experiment stopped	52,389 ♀	,, 5 ,, Dosed
52,391 ♀	,, 6 ,, (2 g.). *Then for 9 weeks (5 g.).* Experiment stopped		

* These rats received faeces which had been sterilised.
The litter of the rats is indicated by the figures in the fourth and fifth places.

appreciably (average 2 g. per week) (see Fig. 1, curve *C* ii), but in 8 out of the total 16 there was a sudden increase in the rate of growth after varying periods of coprophagy (Table I, italics and Fig. 1, curve *C* iii). This growth was not very rapid (average 6 g. per week) but it was steady and continued till the animals weighed about 100–110 g., but never beyond this. Four of the rats were killed while still growing (rats 4,003, 4,005 and 4,007), two others stopped growing, lost weight and died of neuritis (rats 2,001 and 2,011) and the other two remained stationary in weight for several months and then lost weight and died of neuritis (rats 2,004 and 2,005).

No difference is apparent in the improvements due to coprophagy, between those rats which consumed their faeces from the start (litters 1,000, 2,000 and 4,000) and those which received a vitamin-free diet for 1 week first (litters 51,000 and 52,000).

From this experiment it appeared that coprophagous rats on cooked diets deprived of the entire vitamin B complex acquired, through the eating of their faeces, sufficient of the complex to prolong their lives for considerable periods. In all the cases investigated death finally occurred as a result of neuritis due to vitamin B_1 deficiency.

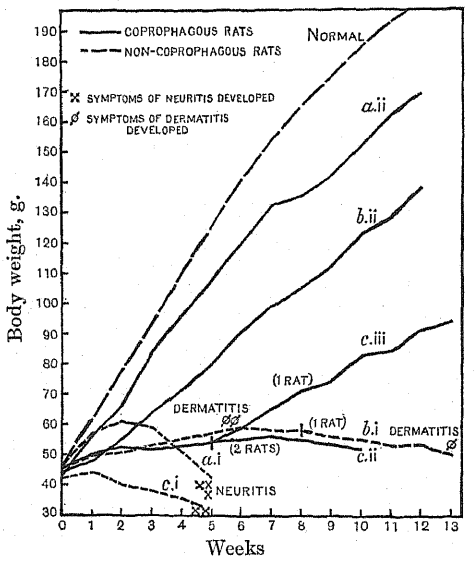


Fig. 1. *Exp.* 3. The growth of coprophagous rats on diets: (*a*) deprived of vitamin B_1, (*b*) deprived of vitamin B_2, (*c*) deprived of the entire vitamin B complex, compared with that of non-coprophagous controls on the same diets, and with normally growing rats.

The curves are in each case, until the vertical lines, averages for 3 rats, 2 males and 1 female.

Exp. 2. The quantities of faeces excreted and consumed by rats deprived of the vitamin B complex.

In these experiments (litters 2,000 and 4,000) two series of rats received a diet devoid of the vitamin B complex. Records were kept of the dry weights of faeces excreted and fed and of the amounts which they failed to eat (see Table II). The percentages of faeces consumed varied from 67 to 77 % in one litter, from 90 to 100 % in the other, the average consumption for any week never fell below 44 %. The growth, expressed as weekly weight increase, is not apparently related to the amount of faeces eaten during the whole time of the experiment.

In Table II are also shown the amounts of faeces eaten during two successive periods in the histories of 7 of the rats, in the first of which they were losing or stationary and in the second gaining in weight. For 3 rats where consumption was low in the first period (57–69 %) it was slightly raised in the second (71–73 %). For the others consumption was high in the first period (77–100 %) and was not increased in the second. It cannot therefore

be said that the growth was due to an increase in the amount of faeces consumed, but the figures do perhaps indicate that growth was only possible when more than about 70 % of the faeces excreted were being eaten.

After the first few weeks of coprophagy the dry weight of the faeces excreted became very high, rising to as much as 1·6 and 1·8 g. daily, whereas rats receiving the same diet adequately supplemented with B-vitamins, but in which coprophagy was prevented, excreted only 0·4–0·5 g. daily. This increase in amount is probably the result of the accumulation of undigestible products of metabolism which are continually re-ingested.

Table II. *Growth of rats and percentage of faeces consumed on a diet deficient in the vitamin B complex when coprophagy was allowed.*

The figures are given for the whole experiment, for several weeks when weight was being lost or was stationary, and for the immediately ensuing weeks when weight was increasing.

	Whole experiment			Period of loss of weight			Period of gain of weight		
Rat No.	Weeks	Av. weekly growth g.	% of faeces eaten	Weeks	Av. weekly growth g.	% of faeces eaten	Weeks	Av. weekly growth g.	% of faeces eaten
2,001 ♂	14	3·3	77	4	−2·5	77	4	4·5	74
2,004 ♀	16	4·0	71	3	−2·7	59	3	6·0	71
2,005 ♂	16	3·9	67	3	−3·3	69	3	9·3	72
2,011 ♂	16	3·7	69	5	−1·0	57	5	5·2	73
	Average	3·7	71						
4,002 ♂	13	1·8	93	—	—	—	—	—	—
4,003 ♀	19	4·2	90	3	−1·0	95	3	10·1	90
4,004 ♀	9	0·6	97	—	—	—	—	—	—
4,005 ♂	19	4·0	98	3	1·7	100	3	6·6	96
4,006 ♂	9	0·0	100	—	—	—	—	—	—
4,007 ♀	19	2·0	98	5	0·4	100	5	5·2	93
	Average	2·1	96						

Exp. 3. The effect of coprophagy in rats deprived of vitamins B_1 and B_2 respectively.

In this experiment (litters 45,000, 51,000, 52,000 and 66,000) the effect of coprophagy in rats deprived of either of vitamin B_1 (series (*a*)) or of vitamin B_2 (series (*b*)) only was investigated.

The experiment lasted 10 weeks. Table III shows the increase in weight during the first 5 and second 5 weeks respectively and the average weekly growth over the whole period for these rats and for their non-coprophagous litter-mates. Growth figures for rats from the same litters, deprived of the whole vitamin B complex (series (*c*)) are shown for comparison. In Fig. 1 are shown the average growth curves of these rats.

Series (*a*). The weight increase of the coprophagous rats receiving vitamin B_2 in their diet (as F5A) but deprived of vitamin B_1, approached the normal, being 64 g. (average) in the first 5 weeks, falling to 46 g. (average) in the second 5 weeks. Control litter-mates, prevented from coprophagy, grew for 2 weeks then lost weight and all developed neuritis by the 5th week (see Fig. 1, curves *a* i and *a* ii). The weight increase during the second 5 weeks

gave the more accurate measure of the vitamin B_1 obtained from the faeces owing to the exhaustion of reserve stores of vitamin B_1, which were sufficient to allow the growth of the controls for 2 weeks. In a further experiment with rats receiving a diet containing vitamin B_2, in which coprophagy did not start till the body weights were stationary, weight increase during the first 5 weeks of coprophagy was 55 g. (average).

These experiments indicate that enough vitamin B_1 was obtained from the faeces to prevent neuritis and support fairly good growth.

Series (*b*). The coprophagous rats receiving vitamin B_1 in their diet (as YE) but no vitamin B_2 increased in weight during the first 5 weeks by 34 g. (average) during the second 5 weeks by 41 g. (average). The control rats prevented from coprophagy, grew an average of 12 g. in the first 5 weeks, by

Table III. *Exp. 3. Growth of coprophagous rats and their non-coprophagous controls on diets* (*a*) *deprived of vitamin* B_1, (*b*) *deprived of vitamin* B_2, (*c*) *deprived of the entire vitamin B complex.*

Coprophagous rats				Non-coprophagous rats		
	Weight increase (g.)				Weight increase (g.)	
Rat No.	1st 5 weeks	2nd 5 weeks	Average weekly	Rat No.	1st 5 weeks	
(*a*) Diet complete except for vitamin B_1:						
45,338 ♂	68	51	12	45,333 ♂	+11, −15 in 32 days.	Neuritis
45,339 ♂	68	56	12	45,334 ♂	+15, −16 in 35 days.	Neuritis
45,335 ♀	57	30	9	45,340 ♀	+19, −22 in 35 days.	Neuritis
Average	64	46	11		+15, −18	
(*b*) Diet complete except for vitamin B_2:						
45,336 ♂	25	50	7·5	45,332 ♂	13	Dermatitis in 42 days
51,385 ♂	34	24	6	51,378 ♂	13	Dermatitis in 42 days
45,337 ♀	40	46	9	—	—	
52,392 ♀	39	46	8·5	52,386 ♀	11	Dermatitis in 98 days
Average	34·5	41·5	8		12	
(*c*) Diet complete except for the vitamin B complex:						
51,384 ♂	5	4	1	51,383 ♂	+2, −12 in 33 days.	Neuritis
52,390 ♂	12	−5	1	52,388 ♂	+2, − 4	
52,391 ♀	11	(29)	2 (6)	52,389 ♀	+2, −18 in 30 days.	Neuritis
Average	9	0	1		+2, −11	

the 6th week 2 had developed dermatitis, the weight of the other remained stationary and it developed dermatitis after 14 weeks (see Fig. 1, curves *b* i and ii).

Thus when vitamin B_1 was being supplied in the diet an amount of vitamin B_2 sufficient to prevent dermatitis and allow subnormal growth appears to have been obtained by coprophagy.

Series (*c*). These rats, which were deprived of the entire vitamin B complex, increased in weight by only 9 g. (average) in the first 5 weeks of the experiment. This increase was much less than that observed in either series (*a*) or (*b*). During the next 5 weeks 2 rats continued to show no significant increase in weight though the other started to grow (see Fig. 1, curves *c* i, ii and iii).

Thus when no vitamin B_1-containing yeast concentrate (YE) or vitamin

B_2-containing yeast extract (F5A) was being supplied in the diet, less of each vitamin was obtained through coprophagy.

It was possible that the increased production of vitamins B_1 and B_2 respectively in the rats of series (*a*) and (*b*) was due to some factor common to the materials supplying vitamins B_2 (F5A) and B_1 (YE) respectively, or alternatively, the vitamin B_1 obtained from the faeces might have been increased when vitamin B_2 was present in the diet and the vitamin B_2 when vitamin B_1 was in the diet. To test these theories a further experiment was carried out.

Coprophagous rats (litter 66,000) were given the vitamin B_1 yeast concentrate (YE) autoclaved under the same conditions as the vitamin B_2 yeast extract (F5A) (5 hours at 120°, p_H 4·7). The concentrate now contained no vitamin B_1. These rats, however, grew as well as those receiving the unheated concentrate, 47 g. (average) weight increase in 5 weeks. It was not therefore vitamin B_1 that was stimulating the production of vitamin B_2 and it seems probable that there was some heat-stable factor present in both the preparations from yeast (YE and F5A) which stimulated the production of both vitamins B_1 and B_2 in coprophagous rats.

To summarise Exps. 1–3, the faeces of rats deprived of the vitamin B complex appeared to contain small amounts of vitamins B_1 and B_2. These amounts were in some cases increased so that growth of from 6 to 8 g. per week was allowed, either spontaneously, after varying periods of coprophagy (curve *c* iii, Fig. 1), or when some factor present in autoclaved yeast extracts, but not either vitamin B_1 or B_2, was being supplied in the diet (curves *a* ii and *b* ii, Fig. 1).

Exp. 4. The effect of feeding sterilised faeces to rats.

If the vitamin B complex obtained by coprophagous rats were present in the faeces as the result of synthesis by intestinal flora, the rôle of coprophagy might be explained in three ways: (1) the vitamin B complex might be acquired from the faeces, not having been accessible at the place of synthesis, (2) the faeces might re-infect the gut with the synthesising organism, or (3) they might contain some substance which stimulates the growth in the intestines of the synthesising organism. The effect of feeding sterilised faeces was investigated to test the second hypothesis. A group of rats was fed on faeces sterilised by heating in a moist state for 3 hours at 80° before drying. This degree of heating, which was not enough to destroy the B-vitamins, was found to kill the bacterial flora, cultures only very occasionally showing any growth.

The histories of these rats are shown in Table I, litter 4000. No difference was detected between the rats receiving sterilised faeces (marked with an asterisk) and their controls receiving faeces dried at 37° without previous treatment. The beneficial effect of coprophagy in rats deprived of the vitamin B complex is not therefore due to a constant re-infection of the gut with a vitamin B-producing organism.

Exp. 5. A comparison of the effects of coprophagy in rats on diets containing insoluble starch (uncooked) and on diets containing soluble starch (cooked).

In Table I are given the histories of 5 coprophagous rats on uncooked diets. Three of these rats thrived no better than their non-coprophagous littermates, death occurring as a result of vitamin B_1 deficiency after about 4 weeks. The other 2 rats, together with one of the controls, became refected.

This result is a great contrast to that obtained with rats which were fed with a cooked diet containing soluble starch, and which were always benefited to some extent by coprophagy. It should be noted again that the American workers on coprophagy all used dextrin as their carbohydrate and so avoided refection.

The condition of refection is obscure but it has been shown to depend on coprophagy. It can be transmitted from an affected rat to another by feeding the faeces, and it can only continue if the rats eat their own faeces. Fridericia and his co-workers concluded that micro-organisms in the faeces of refected rats were responsible for the transmission of refection and probably also for its continuance, for transmission was prevented when the faeces were heated for 10 minutes at 100°. Schieblich and Rodenkirchen [1929, 1, 2] have indeed isolated a *Vibrio*-like organism from the faeces and gut of refected rats, which they think to be the responsible agent.

The state of refection has many points in common with that of the coprophagous rats described in this paper. In both cases rats receiving no B-vitamins in the diet remained in health for considerable periods and even exhibited growth. This was much more marked in the refected rats, however. In both cases the state was induced and maintained in rats by the eating of their own faeces, though refected animals only ate a small quantity, the others a large proportion (see Exp. 2).

The two phenomena are however different. Refection only occurred in rats receiving diets containing raw starch and ceased if the diet were cooked, whereas the prolongation of life and the growth observed in these experiments only occurred in rats receiving diets in which the starch had been rendered soluble by cooking.

It is however possible that the organism supposed to be responsible for refection might be also functioning in the coprophagous non-refected rats, though unable to synthesise as much vitamin B complex when the diet was cooked. An experiment was therefore carried out in which the cooked diet of 5 growing coprophagous rats was changed to an uncooked diet.

These were rats 45,338 and 45,335 receiving vitamin B_2 but no vitamin B_1, rats 45,336 and 45,337 receiving vitamin B_1 but no vitamin B_2, and rat 52,391 receiving no vitamin B complex. After the first week on the new diet all lost weight steadily, their condition became worse and refection did not develop.

The same organism did not therefore appear to be responsible for the possible synthesis of the B-vitamins in the two conditions. If synthesis by

micro-organisms in the gut is responsible for the supply of the vitamins to refected rats and to the unrefected coprophagous rats respectively, it appears that those causing refection flourish in a gut filled with raw starch, and the others in one containing soluble starch.

Discussion.

The following conclusions may be drawn from these experiments as to the origin of the vitamin B complex which enables coprophagous rats on diets deficient in the complex, but containing soluble starch, to live and thrive.

It is improbable that the B-vitamins were obtained from the body stores excreted in the faeces, for firstly, there was wastage of the vitamins in the unconsumed faeces and even when faeces passed during the 1st week of vitamin B depletion were discarded, there was no lessening of the good effect of coprophagy (a result in contradiction to that obtained by Dutcher and Francis [1923]). Secondly, it frequently happened that whereas at the start of an experiment coprophagous rats only maintained their weights, after several weeks, when their body stores would be depleted, they started to grow, without any increase in consumption of faeces.

Synthesis of the vitamin B complex in these animals is therefore indicated, and this theory is supported by the fact that the amounts of the B-vitamins available for the coprophagous animal appeared to be increased by the presence in the diet of a factor, contained in autoclaved yeast extracts, but not one of the known B-vitamins (Exp. 3). This is difficult to explain except on the assumption that synthesis was being stimulated by this factor.

It seems probable that the synthesis was carried out by bacteria. This was not proved however, and an experiment designed to show whether the benefit of coprophagy was due to reinfection of the gut with bacteria gave negative results. Also it is difficult to explain by this theory the very steady and consistent growth obtained in the two series of animals (*a*) and (*b*) in Exp. 3, which were deficient in vitamins B_1 and B_2 respectively. For it is hard to suppose that the micro-organisms present in different animals would always produce the same amounts of these vitamins, or that they would be eliminated in the same amounts.

The exact rôle of coprophagy is not apparent. It may be that the vitamins were synthesised by bacteria in the intestines, but that they were not liberated here but only when the bacteria were digested higher up in the gut after re-ingestion of the faeces, or the faeces may contain some factor which stimulates the growth of bacteria.

No connection could be traced in these experiments between the process whereby these coprophagous rats, receiving diets containing soluble starch, obtained their B-vitamins and that whereby refected rats obtained their supplies. If both processes are based on a bacterial synthesis, it appears that different bacteria are involved in each.

SUMMARY.

1. A quantitative method of controlling the degree of coprophagy in rats, by collecting, drying and feeding the faeces is described.

2. The life of rats receiving diets deficient in the vitamin B complex or its constituents and containing soluble starch was prolonged considerably when they ate their faeces, and in some cases growth also took place. This was not prevented by a preliminary week of depletion during which coprophagy was not permitted.

3. From 44 to 100 % of their faeces was consumed by the individual rats. Growth did not occur when less than 70 % was being consumed, but it was not necessarily proportional to increased consumption.

4. The above facts are most easily explained by the theory that in coprophagous rats there is a synthesis of the B-vitamins, probably by bacteria in the lumen of the gut.

5. This synthesis was stimulated by the presence in the diet of a heat-stable factor, contained in autoclaved yeast extracts and differing from the recognised B-vitamins.

6. The rôle played by the re-ingested faeces is not evident. The efficacy of sterilised faeces showed that the action was not due to the re-infection of the gut with a synthesising organism. It may be that the B-vitamins are obtained directly from the faeces, or the faeces may contain a substance which stimulates the growth of synthesising bacteria already established in the gut.

7. When the rats were fed on uncooked diets containing raw starch, coprophagy did not benefit them unless refection occurred. Changing from a cooked to an uncooked diet resulted in the development of symptoms of deficiency of the B-vitamins.

8. Refection is a condition distinct from that observed in coprophagous rats fed on diets containing soluble starch. It only occurred in rats fed on diets containing raw starch. No connection between the two conditions could be traced.

My thanks are due to Dr H. Chick for advice and criticism throughout the course of this work and to Miss D. Graetz for help with the care of some of the experimental animals.

REFERENCES.

Bechdel, Honeywell, Dutcher and Knutsen (1928). *J. Biol. Chem.* **80**, 231.
Cooper (1914). *J. Hygiene*, **14**, 12.
Dutcher and Francis (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 189.
Fridericia, Freudenthal, Gudjonnsson, Johansen and Schonbye (1927). *J. Hygiene*, **27**, 70.
Gaglio (1919). *Il Policlinico*, **26**, 1381.
Heller, McElroy and Garlock (1925). *J. Biol. Chem.* **65**, 255.
Kennedy and Palmer (1928). *J. Biol. Chem.* **76**, 607.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.

McCollum, Simmonds and Becker (1925). *J. Biol. Chem.* **63**, 547.
Muckenfuss (1918). *J. Amer. Chem. Soc.* **40**, 1606.
Peters (1924). *Biochem. J.* **18**, 858.
Portier and Randoin (1920). *Compt. Rend. Acad. Sci.* **170**, 478.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Roscoe (1927). *J. Hygiene*, **27**, 103.
—— (1930). *Biochem. J.* **24**, 1754.
Scheunert, Schieblich and Rodenkirchen (1929). *Biochem. Z.* **213**, 226.
Schieblich (1929). *Biochem. Z.* **207**, 458.
—— (1930, 1). *Biochem. Z.* **220**, 394.
—— (1930, 2). *Biochem. Z.* **225**, 212.
—— and Rodenkirchen (1929, 1). *Biochem. Z.* **213**, 234.
—— —— (1929, 2). *Biochem. Z.* **213**, 245.
Steenbock, Sell and Jones (1923). *J. Biol. Chem.* **55**, 411.
—— —— and Nelson (1923). *J. Biol. Chem.* **55**, 399.
Walle, Van der (1922). *Biochem. J.* **16**, 713.

CCXXIV. OXIDATION-REDUCTION POTENTIALS OF *STAPHYLOCOCCUS* CULTURES.

II. EFFECT OF BACTERIOPHAGE.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

(*Received October 31st, 1931.*)

In the presence of the appropriate bacteriophage the fall in potential of *B. dysenteriae* (Shiga) cultures is completely inhibited whilst in the case of *B. coli* [Hewitt, 1931, 1] the fall in potential is merely delayed. These effects on the oxidation-reduction potential follow the effect on the proliferation of the bacteria, since growth is almost completely inhibited by bacteriophage in the case of *B. dysenteriae* (Shiga) but is merely delayed in the case of *B. coli*. It was concluded therefore that the effect of bacteriophage is merely to inhibit or delay the fall in potential associated with the growth and metabolic activities of the bacteria.

The oxidation-reduction potentials observed in *Staphylococcus aureus* cultures have already been described [Hewitt, 1930] and this paper describes the effect of bacteriophage on the potentials of the cultures.

General methods.

The general methods and apparatus used were those described previously [Hewitt, 1931, 2]: 0·1 cc. of a 24-hour broth culture of *S. aureus* was inoculated into 8 cc. of medium, and the potentials were measured periodically in each experiment.

The sample of bacteriophage and sensitive strain of *S. aureus* used were kindly provided by Dr C. H. Andrewes. Before use the bacteriophage was "subcultured" several times by adding it to 6-hour broth cultures of *S. aureus*, incubating for a further 18 hours and then filtering through Seitz filters. In each experiment 0·25 cc. of phage filtrate was added to each 8 cc. of culture.

Results.

The oxidation-reduction potential curves of the cultures without bacteriophage were substantially the same as those described previously [Hewitt, 1930] with a different strain of *S. aureus*. The effect is described below of the addition of bacteriophage to the cultures both at the time of inoculation with the organisms and after 5 hours' incubation.

Stationary aerobic cultures (Fig. 1). The potential during the first 6 hours' incubation fell as rapidly in the presence of bacteriophage as in its absence, but after this, whereas the potential in the culture without bacteriophage continued to fall and reached the level E_h — 0·18 v., in the phage culture the potential rose slightly and did not at any time fall below the level — 0·10 v. It will be seen later that these effects, similar in kind but exaggerated in degree, are observed also in the case of aerated cultures. When bacteriophage

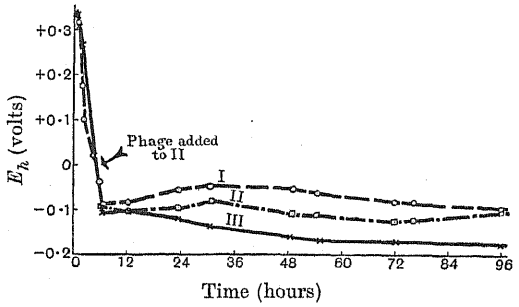


Fig. 1. Stationary aerobic broth cultures of *Staphylococcus aureus*. I. Phage added at beginning of incubation. II. Phage added after 5 hours' incubation. III. No phage added.

was added to a *Staphylococcus* culture after 5 hours' incubation the potential of the culture continued to fall for a time but then ceased to fall, and the lowest level of potential reached (— 0·12 v.) was intermediate between that of the culture without bacteriophage and that of the culture to which bacteriophage had been added at the beginning of incubation. At the end of the first 12 hours' incubation marked growth was visible in the culture not containing bacteriophage; no growth was visible in the culture to which bacteriophage had been added at the beginning of incubation, and there was very faint growth in the culture to which bacteriophage had been added after 6 hours' incubation. In the culture to which bacteriophage had been added at the beginning of incubation a faint trace of growth was observed first after 30 hours' incubation and it will be seen in Fig. 1 that after this period a slight fall in potential occurred. The initial fall in potential had occurred without marked proliferation of the organisms. A similar phenomenon was observed in the case of *B. coli* but not with *B. dysenteriae* (Shiga) [Hewitt, 1931, 1]. It was observed that cultures to which bacteriophage had been added were more acid than those not containing bacteriophage after 6 days' incubation. This will be commented upon later.

Aerated cultures (Fig. 2). It was found in aerated cultures, as in stationary aerobic cultures, that the presence of bacteriophage had no appreciable effect on the potential during the first 5 hours' incubation, but after this period the potential began to rise rapidly in the cultures containing bacteriophage whilst in the cultures without bacteriophage the potential continued to fall slowly. After 24 hours' incubation the potential in the phage culture had fallen again and at this period growth was first visible. In the culture not containing

bacteriophage growth was observed after only 6 hours' incubation. In the culture to which bacteriophage was added after 5 hours' incubation the potential continued to fall for a time but then rose rapidly and later fell again. This second fall in potential was accompanied by proliferation of the bacteria. The hump-shaped potential-time curves appear to be characteristic of the aerated phage cultures, and are associated with changes in the proliferation of the organisms.

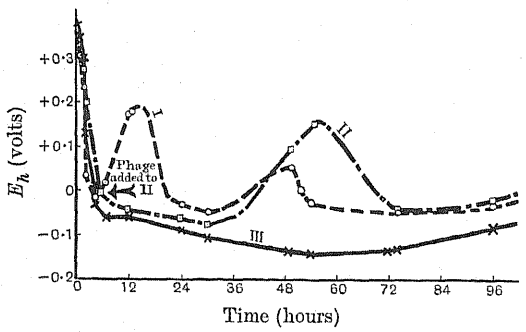


Fig. 2. Aerated broth cultures of *S. aureus*. I. Phage added at beginning of incubation. II. Phage added after 5 hours' incubation. III. No phage added.

Discussion.

The effects of bacteriophage on the oxidation-reduction potentials of *S. aureus* are entirely different from the effects observed with *B. dysenteriae* (Shiga) but are similar in some respects to those observed with *B. coli* [Hewitt, 1931, 1]. In the case of the Shiga bacillus proliferation of the organisms and the associated fall in potential are almost completely inhibited in the presence of bacteriophage, but in the case of *B. coli* proliferation and the fall in potential are merely delayed. On the whole the effect of bacteriophage on the potentials of cultures follows the effect on the growth of the organisms. An exception to this correlation between growth and the fall in potential occurs in the case of stationary aerobic 1 % glucose broth cultures of *B. coli* containing bacteriophage in which the potential shows an initial fall despite absence of bacterial growth. In the case of *S. aureus* also there is an initial fall in potential of cultures in the presence of bacteriophage without appreciable proliferation of the bacteria both in aerobic and aerated plain broth. In aerated cultures containing bacteriophage the potential rises rapidly after this initial fall but later it falls again when visible growth becomes apparent in the culture. The initial fall in potential appears to be due to the enzymic activities of non-proliferating bacteria, but the subsequent fall occurs only when marked growth of the culture takes place. This succession of alterations in potential, namely a fall, rise and fall, gives the potential-time curves of aerated cultures a typical hump-shaped appearance. In stationary aerobic cultures the potential-time curves follow a similar course to those of aerated cultures

except that the rise in potential after the initial fall is much less marked since the oxygen supply is more restricted.

There is a fall in potential during the lysis of *M. lysodeikticus* by lysozyme [Hewitt, 1931, 1] and it was tentatively suggested that this fall in potential might be due to the activity of the enzymes of the lysing bacteria. The fact that in cultures of *S. aureus* containing bacteriophage an initial fall in potential is observed without visible growth of the bacteria suggests that the enzymes of non-proliferating bacteria may be producing this fall in potential and lends some support to the hypothesis that the fall in potential during lysis of *M. lysodeikticus* is a manifestation of the enzymic activity of the lysing bacteria.

After several days' incubation *Staphylococcus* cultures containing bacteriophage are frequently more acid than the cultures not containing bacteriophage. This observation may be accounted for by the fact that during the initial phases of growth bacterial cultures in general become acid owing to production of fatty acids *etc.*, but in later stages of growth of some organisms the reaction becomes more alkaline owing to the oxidation of the acids to carbonates. The more acid reaction of many cultures containing bacteriophage may be due to normal occurrence of the acid phase of metabolism and inhibition by the bacteriophage of the subsequent alkaline phase. This hypothesis is supported by the observation that in *S. aureus* cultures the initial fall in potential occurs in the presence of bacteriophage but the subsequent course of the potential-time curve is altered when bacteriophage is present.

From experiments on *B. coli* Young [1929] concludes that oxidations may be effected by organisms which are dead as judged by their reproductive power.

Summary.

1. The potential of *S. aureus* cultures falls initially even in the presence of bacteriophage despite the delay in the proliferation of the bacteria.

2. In cultures containing bacteriophage the potential soon ceases to fall and commences to rise, but a further fall in potential occurs at the time when growth first becomes apparent in the cultures.

3. Apparently the second fall in potential, but not the initial fall, is dependent on the proliferation of the bacteria.

The author is indebted to Dr C. H. Andrewes for the culture and bacteriophage and to Dr R. G. White and Dr E. W. Todd for their encouraging interest.

References.

Hewitt (1930). *Biochem. J.* **24**, 676.

—— (1931, 1). *Biochem. J.* **25**, 1447, 1452, 1641.

—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry. (London County Council.)

Young (1929). *Biochem. J.* **23**, 831.

CCXXV. SYNTHESIS AND CRYSTAL SPACINGS OF CERTAIN LONG-CHAIN PARAFFINS, KETONES AND SECONDARY ALCOHOLS.

By STEPHEN HARVEY PIPER, ALBERT CHARLES CHIBNALL, SYDNEY JOHN HOPKINS, ALFRED POLLARD, JAMES ANDREW BUCHAN SMITH and ERNEST FRANK WILLIAMS.

From the Wills Physical Laboratory, University of Bristol, and the Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received October 31st, 1931.*)

Introduction.

It has long been known that plant waxes contain many substances, notably paraffins and long-chain primary alcohols, besides true waxes such as ceryl cerotate. Attention has been drawn recently to the possible path of metabolism in the plant of some of these substances by Channon and Chibnall [1929] who found that the wax fraction of cabbage leaves (which they refer to as the crude hydrocarbon) consisted chiefly of *n*-nonacosane and 15-nonacosanone. It was suggested by them that paraffins and ketones of this type might arise from the lower fatty acids by condensation and reduction in the following way:

$$\begin{matrix} R_1COOH \\ + \\ R_2COOH \end{matrix} \rightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CO \rightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CH_2.$$

Unfortunately, in the case of the cabbage ketone and paraffin, this postulates the presence of pentadecoic acid at some stage in the synthesis, and as acids containing odd numbers of carbon atoms have not yet been satisfactorily identified in nature, it was felt that the question of the immediate precursor of the ketone should be left over until further experimental evidence was available.

During the past two years we have been investigating the composition of the ethereal extract of numerous forage grasses and, as these all contain a high proportion of wax material, an opportunity has thus been afforded of obtaining more of the required evidence.

We were encouraged to undertake this work by the success attained by Francis, Piper and Malkin [1930] in their researches into the constitution of the long-chain fatty acids occurring in natural products. These authors obtained their standardising data from synthetic fatty acids of 14–26 carbon

atoms, and from mixtures of these. They concluded that the identity of these fatty acids could only be established with certainty when the M.P. and 001 spacings of two crystal modifications were known. They were able to show quite definitely that the naturally occurring acids, usually referred to as arachidic, lignoceric, cerotic and montanic acids, were all mixtures of *n*-fatty acids. Furthermore, the crystal spacings obtained enabled them in certain favourable cases to deduce the probable composition of the mixture.

In the case of the plant waxes the X-ray method of analysis has again proved strikingly successful and the constitution of the main components, *e.g.* paraffins, ketones, primary alcohols, secondary alcohols and fatty acids, has been suggested with such certainty that confirmation by appropriate synthesis has been relatively easy. To attain this result we have had not only to perfect the methods of separating the various components of the waxes, but also to prepare highly pure synthetic products, especially paraffins, in order to have accurate standards of comparison. The present paper, which deals with these synthetic products and their X-ray analysis, is therefore a necessary forerunner to further papers dealing with the components of the various plant waxes.

EXPERIMENTAL.

Preparation of fatty acids.

(The M.P.'s in brackets refer to Francis, Piper and Malkin [1930].)

Kahlbaum's *myristic* and *palmitic* acids were recrystallised four times from large volumes of acetone; yield about 25 %, M.P. 54° and 63·0° respectively (54°, 63·1°).

Pentadecoic acid. 100 g. of Eastman Kodak acid (M.P. 48·5°) was fractionated five times by distillation *in vacuo* and after repeated crystallisation from light petroleum (B.P. under 40°) yielded 23 g. of pure acid; M.P. 52·5° (52·1°).

Stearic acid. Prepared by saponification of a specimen of ethyl stearate (iodine value 0·8) kindly supplied by Prof. Hilditch, which had been prepared by hydrogenation of ethyl oleate; M.P. 70·0° (70·1°).

Eicosanic acid. Prepared from Kahlbaum's erucic acid by heating with potassium hydroxide [Levene, West and Van der Scheer, 1915]. The crude acid was converted to the ethyl ester and fractionated at 206°/2·5 mm. After crystallisation from acetone the ester melted at 41–41·5° (41°) and the acid after repeated crystallisation from alcohol at 75–75·2° (75·2°).

Heneicosanic acid. Ethyl eicosanate was reduced to *n*-eicosanol by means of sodium in dry ethyl alcohol at 130°; yield 80 %, M.P. 65–65·5°. This was converted into the iodide (M.P. 41–42°) by heating with iodine and red phosphorus and hence to the nitrile by boiling overnight with potassium cyanide in absolute alcohol. Without isolation the nitrile was hydrolysed to heneicosanic acid by heating with excess of aqueous sodium hydroxide; yield 75 %; M.P. after repeated crystallisation from acetone and alcohol 74·2–74·6° (75·2).

Synthesis of ketones.

Symmetrical ketones. These have recently been prepared on a large scale in excellent yield by Grün, Ulbrich and Krczil [1926] who heated the requisite fatty acids in an iron vessel. In the present research the method of Gluud [1919] was first tried, as it seemed more applicable to small quantities of material. The barium soaps of the fatty acids were heated in a hard glass retort under reduced pressure, and the ketone, which slowly distilled over, was crystallised twice from alcohol. 12-Tricosanone (laurone), 15-nonacosanone and 16-hentriacontanone (palmitone) were prepared in this way in yields of 50–60 %. It was later found that improved yields could be obtained by a slight modification of the method of Grün *et al.* as follows.

Myristic acid (15 g.) was heated in a tall hard glass vessel to 285°. A loosely fitting sheet iron ring pierced with numerous holes had been previously placed in the vessel and was of such height that it was just covered by the molten acid. The melt was stirred by a glass paddle rotating within the ring, and after about 2 hours there was a sudden evolution of gas, with increase in viscosity of the liquid and considerable frothing. Heating was continued for a further 2 hours, when evolution of gas ceased. The melt was poured out of the tubes, cooled and then extracted with hot 95 % alcohol. The yellow solution was decolorised with charcoal, and on cooling the 14-heptacosanone (myristone) crystallised out. One recrystallisation from hot alcohol was sufficient to give a pure product; yield 9·7 g. or 75 %. 18-Pentatriacontanone (stearone) was also prepared by this method, the reaction temperature being 300–305°. The melting-points of these ketones and their oximes were in agreement with those of Grün *et al.* [1926] and of Channon and Chibnall [1929]. The oxime of 15-nonacosanone melted at 52–53°.

The following three unsymmetrical ketones were synthesised by the method used by Channon and Chibnall [1929] for the synthesis of 14-nonacosanone which is based on the synthesis of 6-ketostearic acid by Robinson and Robinson [1925]. The oxime of 14-nonacosanone has since been prepared; M.P. 45·5–46°.

12-Nonacosanone, $CH_3.(CH_2)_{10}.CO.(CH_2)_{16}.CH_3$. Ethyl palmitate, B.P. 152–155°/1 mm., was reduced with sodium in dry ethyl alcohol to *n*-hexadecanol, which was purified by distillation at 153–156°/1 mm., M.P. 50–51°. This was converted to the iodide by heating with iodine and red phosphorus. Sodium (5·5 g.) was dissolved in 90 cc. of absolute alcohol, ethyl acetoacetate (47 g.) added and finally *n*-hexadecyl iodide (82 g.). The mixture was boiled for 5½ hours, poured into 1500 cc. of water and the resulting ethyl 2-acetyl-*n*-octadecoate, $CH_3.(CH_2)_{15}.CHAc.CO_2.Et$, extracted with ether. It was fractionated twice and the fraction boiling at 189–195°/1·5 mm. collected; yield 56 g. (Found: C, 74·3; H, 11·8. $C_{22}H_{42}O_3$ requires C, 74·6; H, 11·9 %.) This ester (56 g.), dissolved in 300 cc. dry ether, was added to emulsified sodium (3·5 g.) suspended in 300 cc. dry ether and the mixture warmed until the sodium had dissolved. The mixture was then cooled on ice and dodecoyl chloride

(37·7 g.) in 80 cc. dry ether added. After standing for one hour the mixture was boiled for 10 minutes, cooled, the sodium chloride removed by washing with water and the ether removed by evaporation. The resulting oily 12-keto-13-acetyl-13-carbethoxy-*n*-nonacosane was dissolved without purification in 1400 cc. absolute alcohol. 5 % aqueous potassium hydroxide was then added until there was a slight turbidity and the mixture kept 4 days. Sufficient strong potassium hydroxide was then added to make the concentration 5 % and the solution boiled for 3 hours. The alcohol was evaporated and the solid residue extracted 6 times with hot acetone. The extracts on cooling deposited crystals, which were dissolved in light petroleum; the filtered solution was concentrated, and treated with two volumes of acetone. 12-Nonacosanone (20 g.) was precipitated. It was crystallised from 95 % alcohol (charcoal), M.P. 74·5°. (Found: C, 82·4; H, 13·3. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 47–47·5°.

10-Nonacosanone, $CH_3.(CH_2)_8.CO.(CH_2)_{18}.CH_3$. By the method given above sodium (1 g.), alcohol (16 cc.), ethyl acetoacetate (7·5 g.) and *n*-octyl iodide (10 g.) gave 5·7 g. of ethyl 2-acetyl-*n*-decoate, a colourless oil, B.P. 140–141°/5 mm. Eicosanoyl chloride (6·4 g.) in dry ether (25 cc.) was added to ethyl sodio-2-acetyl-*n*-decoate (5·14 g.) in dry ether (25 cc.). The condensation product was saponified as before and yielded 2·4 g. of 10-nonacosanone. Recrystallised from light petroleum (B.P. 40–60°) it melted at 74·3–74·8°. (Found: C, 82·6; H, 13·8. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 49·5–50°.

9-Nonacosanone, $CH_3.(CH_2)_7.CO.(CH_2)_{19}.CH_3$. Sodium (1 g.), alcohol (16 cc.), ethyl acetoacetate (7·5 g.) and *n*-heptyl iodide (10 g.) gave 6·3 g. of ethyl 2-acetyl-*n*-nonoate, a colourless oil, B.P. 122°/2 mm. Ethyl sodio-2-acetyl-*n*-nonoate (4·6 g.) condensed with heneicosanoyl chloride (6·3 g.) in dry ether yielded 2·2 g. of 9-nonacosanone. Recrystallised twice from light petroleum it melted at 73·5–74·2°. (Found: C, 82·2; H, 13·9. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 48·5–49°.

All these long-chain ketones are fairly soluble in cold and very readily soluble in hot chloroform, benzene and xylene; slightly soluble in cold ethyl acetate, light petroleum (40–60°) and carbon disulphide; and almost insoluble in cold but fairly soluble in boiling acetone, ethyl and methyl alcohol. The oximes are readily soluble in cold light petroleum and in acetone-ether mixture.

Synthesis of secondary alcohols.

These were prepared in the usual way by reduction of the ketones. The ketone (1 g.) was dissolved in boiling amyl alcohol (40 cc.) and sodium (5 g.) slowly added. After 2 hours the mixture was cooled and washed with dilute hydrochloric acid and with water. The solvent was removed under reduced pressure and the residue recrystallised from alcohol (charcoal) and then from light petroleum (B.P. 40–60°). It was sometimes necessary to repeat the reduction. The melting-points (Table VI) of the symmetrical alcohols confirm

those of Grün *et al.* [1926]. The following have not previously been prepared. *15-Nonacosanol*, M.P. 83·6–83·8°, S.P.[1] 83°; *acetate* M.P. 50·5–51°. *14-Nonacosanol*, M.P. 79–79·3°, S.P. 78·4°; *acetate* M.P. 41–42°. *12-Nonacosanol*, M.P. 74–74·5°, S.P. 73°; *acetate* M.P. 44–45°. *10-Nonacosanol*, M.P. 74·8–75°, S.P. 74·2°; *acetate* M.P. 45–45·5°. *9-Nonacosanol*, M.P. 75·3–75·6°, S.P. 74·8°; *acetate* M.P. 44·5–45°.

The alcohols have similar solubilities to the ketones, but are markedly less soluble in cold solvents such as low-boiling petroleum.

Synthesis of higher paraffins.

The paraffins with an odd number of carbon atoms were synthesised by Clemmensen reduction of the corresponding ketones. The following synthesis of *n*-nonacosane, which has not hitherto been prepared, will illustrate the method employed.

n-*Nonacosane*. Zinc wool (30 g.) was amalgamated by allowing to stand in 5 % aqueous mercuric chloride for one hour. It was washed and placed in a flask fitted with a reflux condenser together with 15-nonacosanone (2 g.) and 200 cc. of a mixture of equal volumes of concentrated hydrochloric acid and alcohol saturated with dry hydrogen chloride. The mixture was then boiled until evolution of hydrogen slackened, when the flask was cooled and the aqueous alcohol poured off. Fresh acid mixture was added as many times as was necessary to dissolve all the zinc. A further 20 g. was then added and the operation repeated. The final product was washed with water and recrystallised from alcohol (charcoal). It melted at 62·7–63·0°, and analysis showed it to be a hydrocarbon. (Found: C, 85·2; H, 14·8. $C_{29}H_{60}$ requires C, 85·2; H, 14·8 %.) Paraffins prepared in this way were used in the earlier X-ray experiments, but the pictures obtained were inferior to those of natural paraffins. After various methods of purification had been tried it was found that treatment with sulphuric acid was the most effective. The paraffin was heated with about 6–8 volumes of concentrated sulphuric acid in a test-tube immersed in an oil-bath at 130° for 2 hours. Considerable blackening of the acid occurred. The tube was removed from the bath, the paraffin melt on the surface of the acid allowed to set, and the acid drawn off. Fresh acid was added and the tube replaced. This operation was repeated two or three times if necessary, depending on the amount of charring that occurred. The paraffin was washed repeatedly with hot water, and crystallised from 95 % alcohol (charcoal). The purified product was again treated with concentrated sulphuric acid, when it was usually found that a little darkening still occurred. The treatment was repeated until a sample of sulphuric acid which had remained in contact with the paraffin at 130° for 2 hours showed no darkening. In all cases this treatment raised the melting-point of the paraffin about 0·5°. There was usually a loss during purification of 10–20 %, due to the solubility of the paraffin in the acid and to the

[1] S.P. signifies setting-point.

decomposition of olefines formed during the reduction. (Before treatment with sulphuric acid the *n*-nonacosane had iodine value 1·3 and *n*-pentatriacontane 2·6.) We have proved by appropriate experiments that ketones and alcohols are also quantitatively removed by this treatment. The following are in general agreement with Grün *et al.* [1926] and Kraft [1882]: n-*heptacosane*, M.P. 59·0–59·2°, S.P. 58·5°; n-*hentriacontane*, M.P. 67·6–67·8°, S.P. 67·3°; n-*pentatriacontane*, M.P. 74·4–74·6°, S.P. 74·3°. An earlier sample of *n*-pentatriacontane prepared from another sample of stearic acid, which was thought at the time to be pure, gave the same M.P. (74·6°) but a lower S.P. (74·0°). The X-ray picture was also inferior. This sample was the one used by Garner *et al.* [1931].

The following paraffins were synthesised by the method of Gascard [1921].

n-*Octacosane*. This paraffin has not previously been synthesised. A sample of *n*-tetradecanol was kindly supplied by Prof. Garner. It had been twice distilled, then recrystallised four times from alcohol and finally distilled, M.P. 38–38·5°, S.P. 37·7°. It was converted into the iodide (2·17 g.) which was boiled in sulphur-free xylene (70 cc.) with sodium (0·2 g.) for 3 hours. The cooled solution was washed with water, evaporated to 10 cc. and treated with two volumes of alcohol to precipitate the paraffin (0·54 g. or 36 %). The first treatment with sulphuric acid for 4 hours at 130° caused a little blackening of the acid, the second none at all, M.P. 61·4–61·5°, S.P. 61·1°. This paraffin was also prepared by the method of Backmann and Clark [1927]. Tetradecyl iodide (2·46 g.) was heated at 150° for 2 hours and sodium (1·3 g.) added slowly in small pieces with vigorous stirring. The yield after treatment with sulphuric acid was 70 % of theory. The X-ray picture showed that this preparation was inferior to the former, although the M.P. and S.P. were the same.

n-*Dotriacontane*. *n*-Hexadecyl iodide (1·13 g.) prepared from synthetic *n*-hexadecanol (p. 2074) was boiled in sulphur-free xylene (50 cc.) with sodium (0·2 g.) for 4 hours and the paraffin treated as before; yield 0·16 g., M.P. 69·5–69·7°, S.P. 69·3°. The paraffin prepared from purified natural cetyl alcohol (M.P. 49°) had M.P. 70·3–70·7°, confirming that of Gascard [1921], 70·5°. Kraft [1882] pointed out that natural cetyl alcohol contained some 4–5 % of octadecyl alcohol; the M.P. of the derived paraffin confirms this.

n-*Hexatriacontane*. This was prepared from *n*-octadecyl iodide (0·5 g.) kindly supplied by Dr Malkin, and sodium (0·1 g.) in boiling xylene as before, M.P. 75·7–75·9°, S.P. 75·4°. (Gascard [1921], M.P. 76°.)

The following paraffins were kindly supplied by Prof. Garner [*cf.* Garner, van Bibber and King, 1931]. They had been prepared by electrolysis of the respective fatty acids [Peterson, 1906] and were purified by treatment with sulphuric acid.

n-*Hexacosane*, M.P. 56·4–56·6°, S.P. 56·1° (Gascard [1921], M.P. 56·5°; Garner *et al.* [1931], S.P. 56·1°).

n-*Triacontane*, M.P. 65·6–65·8°, S.P. 65·5° (Gascard [1921], M.P. 65·6–66°; Garner *et al.* [1931], S.P. 65·6°). X-ray analysis showed that this paraffin was not pure, although the M.P. seems correct.

n-*Tetratriacontane*, M.P. 72·5–72·8, S.P. 72·4 (Gascard [1921], M.P. 73·2°; Garner *et al.* [1931], S.P. 72·4°).

These long-chain paraffins are freely soluble at room temperature in low-boiling light petroleum and carbon disulphide [*cf.* Brooks, 1922, p. 581 for solubilities of paraffin wax] but the solubility decreases rapidly with lowering of temperature, and with increase of molecular weight.

For the carbon and hydrogen analyses of the substances mentioned in this paper the usual micro-apparatus of Pregl [1924] with the phosphorus pentoxide absorption tube of Drew and Porter [1928] was first used. The carbon, especially in the case of paraffins, was frequently low, possibly due to the volatility of the substances at high temperature. To obtain accurate and reproducible results it was necessary to modify the apparatus and usual procedure in the following way. The length of the copper oxide column in the tube was increased from 140 mm. as recommended by Pregl to 160 mm., the gauze round that part of the tube containing this oxide being heated to a bright red heat. The actual burning of the compound was carried out rather more slowly than is necessary for the average substance, 70 cc. of oxygen being passed in for this purpose at the rate of not more than 3 cc. per minute, this being followed for "sweeping out" purposes by 100 cc. of air at 4–5 cc. per minute.

Measurement of the crystal spacings.

There are now available large numbers of measurements of the 001 spacings of various long-chain compounds. For any homologous series linear relationships exist between these spacings and the number of carbon atoms in the chain. It is obvious that the spacings of a series once measured will serve as a means of identification. Original work on synthetic paraffins has been carried out by Müller and Savile [1925, 1928, 1930] and Piper *et al.* [1925].

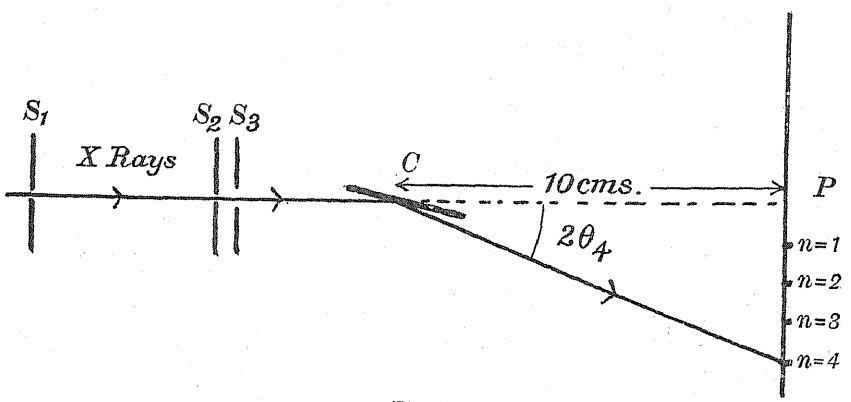


Fig. 1.

Although the technique has often been described it will be convenient to outline the procedure. The substances all crystallise in flakes, and the required planes lie parallel to the flake surfaces. A very thin layer is pressed on to a plate-

glass mount to give a surface of about 1 cm. by 5 mm. wide. The plate is held vertically in a geometric clamp on the spectrograph in such a way that the surface is in the axis of rotation of the table. A narrow beam of X-rays from a Shearer tube of copper anticathode, filtered by a nickel foil, passes through the slits S_1, S_2, S_3, Fig. 1, and falls on the crystals at grazing incidence. The crystal is rocked to and fro through 14° by a cam mechanism, and the radiation it reflects is received on a photographic plate placed 10 cm. away at P. This plate is adjustable for height and is provided with a lead screen allowing successive exposures of strips 5 mm. deep. As many as 16 exposures can be made on the same plate. An exposure takes 30 minutes after which the crystal is turned through 180° and a second exposure is made on the other side. Reflection only occurs at glancing angles θ_1, θ_2, θ_3, *etc.*, determined by the Bragg equation

$$n\lambda = 2d \sin \theta_n,$$

where λ is the wave-length, $n = 1, 2, 3$, *etc.*, the order of the reflection, and d the crystal spacing. With good material and chains of 20 or more carbon atoms 9 or 10 orders can be obtained between $\theta = 0$ and 14°. The trace on the plate consists of a series of lines approximately equally spaced and decreasing in intensity with increasing order, and, since $\sin \theta$ is inversely proportional to d, the lines on the plate are close together for large spacings (long chains) and more widely separated for short chains. The values of θ for as many orders as possible are found, and d calculated. It has to be remembered that the actual position of the reflecting surface is indeterminate, but in front of the axis of rotation of the table. The correction to be applied decreases with increasing order and must be found by successive approximations from the value of d obtained from a high order. As a result the larger the number of orders measured the greater the accuracy attainable.

The spacing of a given specimen of paraffin can, with the apparatus in use, be measured to an accuracy of 0·1 %. However, we have found that different synthetic preparations of the same paraffin, apparently equally pure, and made by the same process, sometimes do not give spacings agreeing to less than 0·2 %. A repetition of the spacings to an accuracy of 0·1 % can probably only be obtained by working with single crystals, and we have not found it profitable to attempt more than 0·25 % except for the synthetic standards.

A spacing-carbon atoms graph for the paraffins is shown in Fig. 2. The points for 20, 21 and 24 carbon atoms are taken from Müller and Savile [1930], the higher members have been freshly determined from the material whose preparation has been described and are shown in Table I. Fig. 2 shows that the paraffins are polymorphous; *n*-hexacosane, for instance, may crystallise in two forms having spacings 34·95 and 31 Å. The particular crystal form adopted depends upon temperature, the length of the chain, and the degree of purity of the specimen.

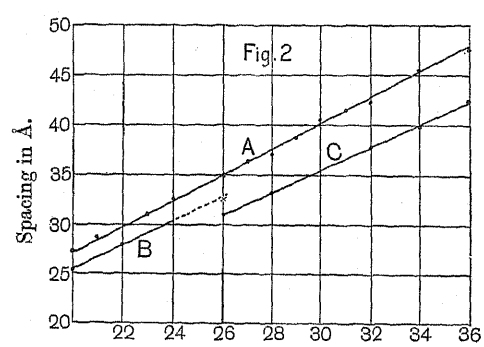
Odd number paraffins of 11 or more carbon atoms normally crystallise with the chain axis vertical to the 001 planes, and the planar spacing is a direct

measure of the length of the molecule. Even number paraffins of 18 or more atoms crystallise in this form at temperatures near the melting-point. These spacings fall on line *A* of Fig. 2. Even number paraffins up to 24 atoms, at normal temperatures, crystallise with the chain axis inclined at a constant angle to the planes. The 001 spacings of this *B* modification are shorter than those of the *A* form and lie on another straight line. *B* crystals are converted to *A* at temperatures very close to the melting-point and resume the *B* form on cooling. Odd number chains of 9 or less also adopt the *B* form under similar conditions [Müller and Savile, 1930].

Table I. *Melting-points and crystal spacings of the paraffins.*

Paraffin	First transition-point on heating	Second transition-point on heating	M.P.	S.P.	First transition-point on cooling	Second transition-point on cooling	Series No.	Crystal spacing in Å. *A*	*B*	*C*	
$C_{26}H_{54}$	51·5–52°	56·0°	56·4–56·6°	56·1°	55·5°	48·3°	C 67	34·95	32·6	31	*B* has only appeared in natural products
$C_{27}H_{56}$	52·8–53°	58·8–58·9°	59·0–59·1°	58·5°	58°	51°	C 22, 23	36·38	—	—	
$C_{28}H_{58}$	57–57·4°	61·2°	61·4–61·5°	61·1°	60·4°	54°	C 114	37	—	33·25	
$C_{29}H_{60}$	57·3–57·5°	63·2°	63·4–63·6°	63·2°	62·2°	55·8°	C 26	38·68	—	—	
$C_{30}H_{62}$	59–59·5°	65–65·3°	65·6–65·8°	65·4°	64·3°	58°	C 69	40·5	—	Not observed	Impure
$C_{31}H_{64}$	62–62·5°	67·2–67·4°	67·6–67·8°	67·3°	66·2°	61·8°	C 125	41·55	—	—	
$C_{32}H_{66}$	65·2–65·4°	69·2–69·4°	69·5–69·7°	69·2°	68·6°	63·9°	C 122	42·33	—	37·8	
$C_{34}H_{70}$	69·2–69·4°	72·2°	72·6–72·8°	72·4°	71·4°	68·5°	C 70, 71	45·5	—	39·86	
$C_{35}H_{72}$	71·8–72°	74·0°	74·4–74·6°	74·1°	73·0°	70·5°	C 28	46·64	—	—	
$C_{36}H_{74}$	73·9–74·1°	75·1–75·3°	75·7–75·9°	75·6°	74·6°	72·5°	B 121	47·5	—	42·33	

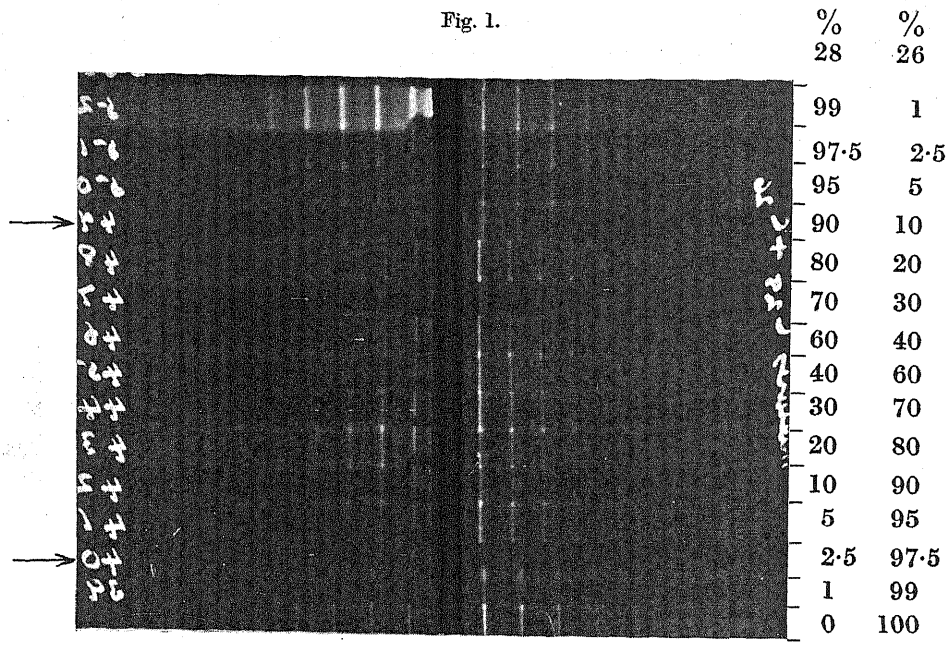
The *A* spacings of the even number paraffins are not so accurate as the remaining measurements. They only occur at temperatures near the M.P. for good crystals, and it is difficult to measure more than three orders.



× = This spacing has not been observed in synthetic hexacosane.

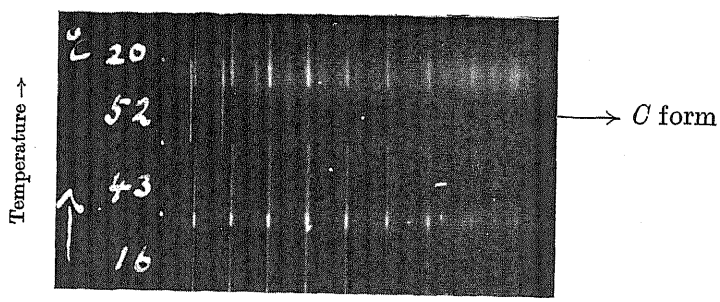
The paraffins that have been most important in this work have 26 or more carbon atoms. If pure these crystallise stably in yet a third modification, *C* of Fig. 2. This form is characterised by a larger tilt of the molecular axis and, of course, by still shorter spacings [Piper and Malkin, 1930]. *n*-Hexacosane can apparently appear in all three forms. We are mainly concerned with spacings lying on the *A* and *C* lines, these are given in Table I. *C* form is converted to *A* at a temperature about 5° below the M.P. Of our specimens hexacosane

Fig. 1.



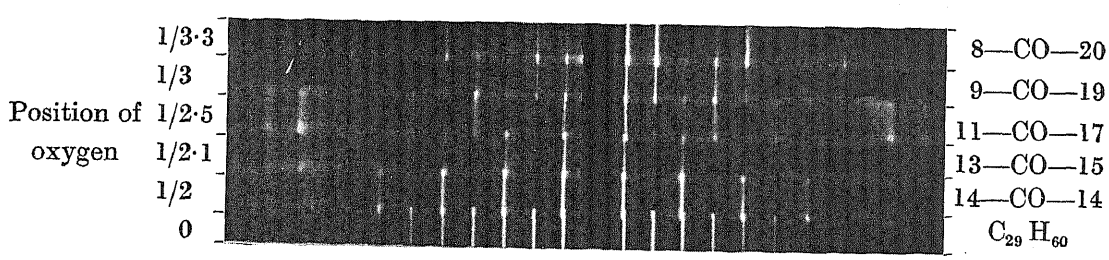
Synthetic mixtures. Changes from *A* to *C* form marked by arrows.

Fig. 2.



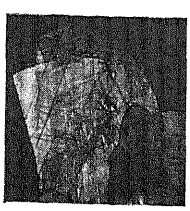
Temperature change in *n*-hexacosane.

Fig. 3.



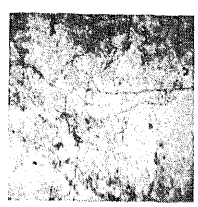
Shearer intensity variation in some nonacosanones.

Fig. 4.



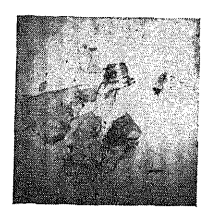
Tetratriacontane. Polarized light.

Fig. 5.



Triacontane.

Fig. 6.



Tetratriacontane.

Unpolarised light.

reverted to *C* on cooling, the others did not, and the reverse change, if it occurs, must be extremely slow at room temperatures. A photograph of hexacosane in both forms is given in Plate VIII, Fig. 2.

The melting- and transition-points of the paraffins.

If the melting-point of a paraffin is carefully determined, as described below, it is possible to obtain not only the true melting- and setting-points but also to determine two transition-temperatures at which a change in crystalline form occurs. The lower of these transition-temperatures is markedly affected by impurities and, in certain cases, is a much more useful guide to the purity of the paraffin than the melting- or setting-point itself. It is necessary therefore to describe in some detail our method of taking these temperatures.

The melting-point apparatus was of the usual glass bulb type and held 80 cc. of sulphuric acid. It was fitted with a standard short stem thermometer which had a spherical bulb of diameter 4 mm. and was graduated in 0·2°. The acid in the bulb was not stirred, and, by using a small flame, it was possible with care to repeat a melting-point with an error of $\pm$ 0·1°. The material was first melted into open capillary tubes of outside diameter about 1 mm. The depth of the melt, which, after sealing, was jerked sharply to the bottom of the tube, was about 2 mm. All melting-points are corrected.

The following observations for *n*-nonacosane will illustrate the method of taking melting-points. The melting-point tube lying alongside the bulb of the thermometer is viewed through a lens giving a magnification of $\times$ 3. At room temperature the material in the tube appears as a white opaque crystalline mass. The temperature is raised rapidly to about 55° and then more slowly (1° per minute). At 57·3–57·5° a transition occurs. The crystalline structure disappears, the material becomes colourless, and an air gap down the centre, in the form of an attenuated inverted cone, is revealed. The temperature is again raised fairly rapidly to 63° and then, still more slowly than before, through the final melting-point. At 63·2° the material once again becomes opaque and the cone structure is no longer seen. Fusion takes place at 63·4–63·6° and the temperature is held within these limits until all the material is melted. The temperature is then allowed slowly to fall; at 63·2° long needles appear throughout the whole molten mass. The heating flame is then withdrawn so that the bath cools a little more rapidly. Between 62·5° and 62·2° the crystalline structure disappears. Contraction during the change of state leads to the formation of the inverted cone-shaped air gap, the rest of the material becoming clear and colourless. At 56° transition takes place quite suddenly to the original white crystalline and opaque form, which is the stable one at room temperatures.

In the case of the longer-chain paraffins, $C_{34}H_{70}$, $C_{35}H_{72}$ and $C_{36}H_{74}$, the transitions are much less marked, and can only be observed if the previous cooling of the material in the tube has been very slow. It is the usual practice in our laboratory to place the tube, when first made, in a bath which is at a

temperature already above the melting-point, and to take the setting- and transition-points on cooling. The melting- and transition-points on heating are taken later. The time required to make the six observations is about 25–30 minutes. To eliminate the personal factor all the melting- and transition-points recorded in this and the two following papers were taken by one observer (A. C. C.).

The melting-, setting- and transition-points of the paraffins between $C_{26}H_{54}$ and $C_{36}H_{74}$ are given in Table I. As we believe that all these compounds (except $C_{30}H_{62}$) are pure the following deductions can be made.

Melting-points. The series $C_{26}H_{54}$ to $C_{32}H_{66}$ lie on a smooth curve (Fig. 3). There is evidence of alternation between $C_{34}H_{70}$, $C_{35}H_{72}$ and $C_{36}H_{74}$. We are therefore not entirely in agreement with the conclusions of Hildebrand and Wachter [1929] who adopted the somewhat dubious course of plotting all the values to be found in the literature, which assumes that all the products have been made from fatty acids of equal purity. The M.P. determined by our method may be some 0·5–0·7° lower than that given by the more usual method in which the temperature of a stirred bath is raised from 3° to 6° per minute. Even so we are unable to explain the high values recorded by Levene, West and Van der Scheer [1915].

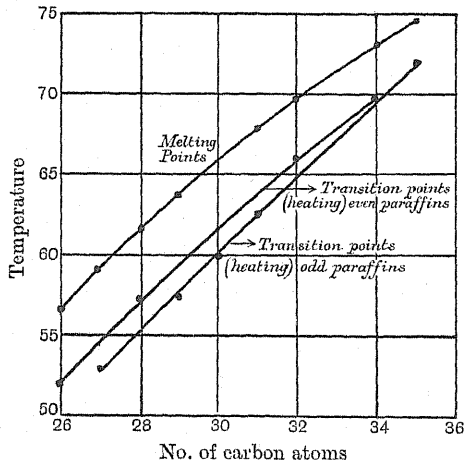


Fig. 3. Melting- and transition-points.

Setting-points. Supercooling was never observed in the case of pure paraffins or mixtures of pure paraffins, and it is unnecessary to leave a nucleus.

First transition-point. Alternations between odd and even numbered chains are observed in the change from the stable low temperature crystalline form to the colourless glass. The temperatures recorded are few in number, but give satisfactorily concordant results. The values are plotted in Fig. 3. Both odd and even series show a convergence of melting- and transition-points as the

chain length increases. Particularly interesting is the position of the transition-temperature of triacontane which falls on the odd number paraffin line. This particular specimen of triacontane will only crystallise with a vertical chain (the *A* form), and, in view of the fact that this is the crystal modification adopted by the odd number paraffins, its position among them appears normal. The even number paraffins crystallising normally with a tilted molecule show, as would be expected (since for them the vertical chain is a less stable form), higher transition-temperatures (Fig. 3). All transition-temperatures on cooling lie on one smooth curve.

Second transition-point. This is often difficult to observe and as it is not apparently affected by impurities it will not be quoted in the tables dealing with mixtures and in further publications. In the future, "transition-point" is to be taken as referring to the first transition mentioned above.

Crystal forms of paraffins.

Microscopic investigation confirms the melting-point data, namely that all the longer paraffins have a transition-point involving a structural change. Plate VIII, Figs. 6 and 4, shows the appearance of some very pure *n*-tetratriacontane under the microscope and between crossed Nicols. The crystals, which are in the *C* modification, appear as rhombs of acute angle 73° and show very clear straight cleavages. There is also a marked double refraction effect noticeable. Plate VIII, Fig. 5 is a specimen of *n*-triacontane in unpolarised light; it is not quite pure and does not display such marked cleavages, also the crystals are very much smaller. They show no marked effect between crossed Nicols. These crystals are in the *A* form. Plate VIII, Figs. 5 and 6 should be compared as showing the difference in appearance in unpolarised light of a fair and a very good preparation of an even number paraffin. Odd number paraffins never take the *C* form, but Müller and Savile [1928] has found single crystals in the *A* form with an acute angle of 66°. The odd number paraffins examined had no definite optic axes, but the crystals were extremely minute and in a confused mass, and there was indication of a slight restoration of the light through crossed Nicols. If the crystals are placed in a heating stage they will all be found to undergo a structural change about 5° below the M.P. both on heating and cooling. A crystal in the *C* form breaks up at this temperature and does not appear to recrystallise with its previous optical properties.

The apparent lack of concordance between these observations and the X-ray data, which indicate transitions only for even number paraffins, is due to the fact that the latter, in our case, deal only with one crystal dimension. The reversible change common to all appears to correspond with one observed by Müller and Savile [1930] near the M.P., and presumably involves a change of cross-section of the cell without affecting the molecular tilt. Such a change should not affect the spacings we photograph. But with the longer even members there is, in addition, a change in tilt at or near this temperature which does not always reverse on cooling, and this change involves an increase in the 001

spacing. A very similar temperature change is found with even number fatty acids, Piper *et al.* [1926].

The heats of crystallisation of the paraffins of 22, 26, 30, 34 and 35 carbon atoms have been measured by Garner, van Bibber and King [1931]. Temperature transitions were found for all these at temperatures about 5° below the M.P. Now it is noticeable that while the heats of transition of 22, 26 and 34 increase uniformly with chain length, the values for 30 and 35 are lower. Prof. Garner kindly allowed us to photograph his material and we found that 22, 26 and 34 were in *B* or *C* form whilst 30 and, of course, 35, were in the *A* modification. Thus both paraffins with a low heat of transition had vertical chains and the other three tilted chains. The spacing and M.P. of triacontane were both high, which suggests contamination by not less than 2·5 % of a longer-chain paraffin. Impurity may not have a marked direct effect on the heat of transition, but it tends to force an even number paraffin to crystallise with a vertical chain, and it is plausible to suggest that part at least of the defect in heat of transition for triacontane represents the difference in energy between the *C* and *A* forms of this paraffin. *C* will, of course, have slightly less energy. Throughout their curves there is no indication of a double break, so the change in tilt occurs simultaneously with the other structural change.

Spacings of mixtures of paraffins.

Assuming that the anomalous results obtained with *n*-triacontane (low heat of transition, anomalous transition temperature, no *C* spacing) were due to lack of purity, we attempted a systematic investigation of the crystallisation of mixed paraffins. These were prepared in the following way. Benzene solutions of known strength were made for each paraffin. To prepare the necessary mixture the requisite aliquot of each benzene solution was pipetted into a small beaker, the benzene removed almost completely on a water-bath, and two volumes of hot alcohol were added. The paraffin mixture crystallises out almost quantitatively on cooling. Since the paraffins have a small residual solubility in the mixed solvent it follows that when one is added only in small amount, *e.g.* 1 %, the mixed crystals may actually contain a little less than this proportion. It is known from recent work on the separation of mixed paraffins by fractional crystallisation that temperature of crystallisation can slightly affect the composition. The spacings for binary mixtures whose chain-lengths differ by one or two carbon atoms are shown in Tables II–V and Figs. 4–7.

Only one 001 spacing occurs for all mixtures unless the components are separated by at least 4 carbon atoms. We commenced with two odd number chains (29 and 31) as the results are not confused with alternative crystal forms. A 1 % addition of 31 in 29 raises the spacing by about 1 %. The spacing continues to rise fairly uniformly with the amount of 31 until at equimolar proportions it is 41·2, approximately the spacing of the *n*-hentriacontane used. The mixed spacing continues to rise to a maximum when 97·5 % of the higher paraffin is present and then falls to that of pure *n*-hentriacontane. This behaviour

appears general for A form spacings; except in one case paraffin impurity up to 1 % increases the spacing whether the contaminating chain is shorter or longer. For 10 % of a shorter chain the spacing approximates to that of the pure substance, but the number of orders decreases. With a longer contaminating chain

Table II. *Melting-points and crystal spacings of mixed paraffins.*

($C_{28}H_{58}$ and $C_{29}H_{60}$.)

Percentage composition		Transition-point on heating	M.P.	S.P.	Transition-point on cooling	X-ray analysis			Remarks
						Series number	Spacing in Å.		
$C_{28}H_{58}$	$C_{29}H_{60}$						*A*	*C*	
100	0	57·2–57·4°	61·3–61·5°	61·1°	53·7°	C 114	37	33·25	The *C* form vanishes at contamination 2·5 %
99	1	56·2°	61·2–61·5°	61·1°	53·2°	B 63	—	33·25	
97·5	2·5	56·0°	61·2–61·5°	61·1°	53·4°	B 64	—	33·3	
95	5	56·0°	61·3–61·5°	61·1°	53·6°	B 65	37·55	—	
90	10	55·2°	61·5–61·7°	61·3°	54·2°	B 93	37·8	—	
80	20	55·0°	61·9–62°	61·5°	53·6°	B 92	38	—	
70	30	55·0°	62·1–62·2°	61·7°	53·5°	B 91	37·85	—	
60	40	55·2°	62·3–62·4°	62·0°	53·8°	B 90	38·1	—	
50	50	55·6°	62·3–62·6°	62·3°	54·0°	B 89	38·5	—	
40	60	55·6°	62·6–62·8°	62·4°	54·2°	B 88	38·45	—	
30	70	56·2°	62·8–62·9°	62·5°	54·4°	B 87	38·45	—	
20	80	56·2°	63–63·2°	62·8°	54·7°	B 86	38·6	—	
10	90	56·8°	63·2–63·4°	63·0°	55·2°	B 62	38·7	—	
5	95	57·2°	63·2–63·5°	63·1°	55·4°	B 61	38·7	—	
2·5	97·5	57·5°	63·3–63·5°	63·2°	55·6°	B 60	38·9	—	
1	99	57·5°	63·4–63·6°	63·2°	55·8°	B 59	38·8	—	
0	100	57·5°	63·4–63·6°	63·2°	56·0°	C 26	38·68	—	

Table III. *Melting-points and crystal spacings of mixed paraffins.*

($C_{29}H_{60}$ and $C_{31}H_{64}$.)

Percentage composition		Transition-point on heating	M.P.	S.P.	Transition-point on cooling	X-ray analysis		Remarks
						Series number	Spacing in Å.	
$C_{29}H_{60}$	$C_{31}H_{64}$							
100	0	57·3–57·5°	63·4–63·6°	63·2°	55·8°	C 26	38·68	
99	1	57·2–57·4°	63·4–63·6°	63·2°	55·4°	C 106	39·12	
97·5	2·5	57–57·5°	63·5–63·7°	63·2°	55·4°	C 96	39·25	
95	5	57–57·2°	63·6–63·7°	63·1°	55·3°	C 97	39·15	
90	10	56·5–57°	63·8–64°	63·3°	55·2–55°	C 98	39·45	
85	15	56·0–56·5°	64–64·2°	63·5°	55–54°*	C 101	39·7	
80	20	56·0–56·5°	64·2–64·4°	63·7°	55–54°*	C 102	39·75	
70	30	56·5–57°	64·6–64·7°	64·1°	55–54°*	C 103	40·55	
60	40	56·5–57°	64·8–65°	64·4°	55–54°*	C 104	40·9	
50	50	57–57·5°	65·3–65·5°	65°	55·5–55°*	C 111	41·2	
40	60	57·5–58°	65·8–66°	65·5°	56–55°*	C 110	41·2	
30	70	59–59·5°	66·2–66·4°	65·8°	57·5–57°*	C 109	41·45	
20	80	60–60·5°	66·6–66·8°	66°	58·5°*	C 105	41·5	
10	90	61–61·5°	67·1–67·3°	66·8°	59·5°	C 108	41·7	
5	95	61·7–61·9°	67·3–67·5°	67·1°	60°	C 100	41·85	
2·5	97·5	61·7–61·9°	67·4–67·6°	67·3°	61·6°	C 99	41·8	
1	99	62·1–62·3°	67·6–67·7°	67·3°	61·7°	C 107	42·0	This increase in spacing is greater than the possible experimental error
0	100	62·3–62·5°	67·6–67·8°	67·3°	61·8°	C 32	41·55	

* = indefinite.

the *A* spacing increases steadily up to 80 % impurity. The *A* spacing then is very sensitive to the addition of small amounts of other paraffins. With *n*-hexacosane and *n*-octacosane mixtures, since both have even number chains, we have to deal with two crystal forms. Both crystallise normally in the *C* form but if more than 2·5 % of the longer chain is added to the shorter, crystallisation occurs only in the *A* form. Up to 10 % of a short chain can be added to a long

one before the *C* modification fails to appear. This is clearly shown in Plate VIII, Fig. 1. The tables show that this is a true generalisation whether the contaminating paraffin has an odd or an even number chain. There is no measur-

Table IV. *Melting-points and crystal spacings of mixed paraffins.*

($C_{26}H_{54}$ and $C_{28}H_{58}$.)

Percentage composition		Transition-point on heating	M.P.	S.P.	Transition-point on cooling	X-ray analysis: Series number	X-ray analysis: Spacing in Å. *A*	X-ray analysis: Spacing in Å. *C*	Remarks
$C_{26}H_{54}$	$C_{28}H_{58}$								
100	0	51·5–52°	56·4–56·6°	56·1°	48·3°	C 67	34·95	31	*C* spacing disappears at more than 2·5 % impurity
99	1	51°	56·5–56·6°	56·0°	48·0°	B 39	—	31	
97·5	2·5	51°	56·5–56·7°	56·0°	47·4°	B 40	35·1	31·6	
95	5	50°	56·5–56·8°	56·0°	47·0°	B 41	35·5	—	
90	10	48·5–49·5°	56·7–57·0°	56·3°	46·5°*	B 42	35·25	—	
80	20	48·5–49·5°	57·2–57·5°	57·0°	46·5°*	B 43	36	—	
70	30	48·5–49·5°	57·8–58°	57·5°	46·5°*	B 44	36·2	—	
60	40	48·5–49·5°	58·4–58·6°	58·2°	47·0°*	B 45	36·55	—	
50	50	49–50°	58·9–59·1°	58·5°	47·4°*	B 97	36·7	—	
40	60	49–50·5°	56·6–56·7°	59·2°	48·8°*	B 46	36·8	—	
30	70	51–52°	60·1–60·2°	60·0°	50·6°*	B 47	37·7	—	
20	80	52–53°	60·4–60·6°	60·1°	52·5°	B 48	37·4	—	
10	90	53–53·4°	61·0–61·1°	60·4°	52·7°	B 49	37·55	33·45	*C* spacing disappears at more than 10 % impurity
5	95	54·5°	61·1–61·3°	60·9°	53·2°	B 50	—	33·35	
2·5	97·5	55–55·5°	61·2–61·4°	60·9°	53·3°	B 51	—	33·3	
1	99	56·5–57°	61·2–61·5°	60·9°	53·4°	B 52	—	33·3	
0	100	57·4°	61·3–61·5°	61·1°	54·0°	C 114	37	33·26	

*=indefinite.

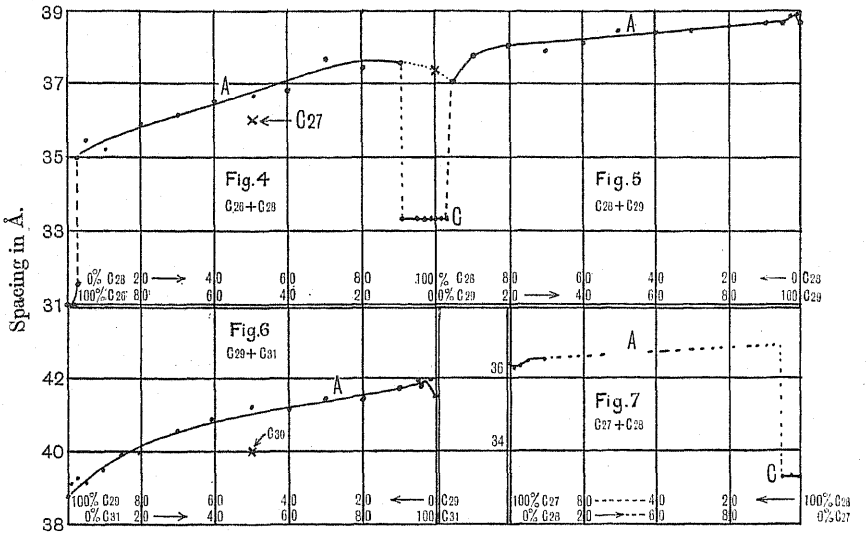
Table V. *Melting-points and crystal spacings of mixed paraffins.*

Composition (equimolar unless stated)	Transition-point on heating	M.P.	S.P.	Transition-point on cooling	Series number	Crystal spacing in Å. *A*	Crystal spacing in Å. *C*	Remarks
97·5 % C_{26}+2·5 % C_{27}	49·4–49·6°	56·4–56·6°	56·1°	48°	B 100	—	30·9	
97·5 % C_{27}+2·5 % C_{26}	52·5–53°	59·0–59·1°	58·6°	—	B 99	36·15	—	
99 % C_{27}+1 % C_{28}	52·5–53°	59·0–59·1°	58·7°	50·6°	B 55	36·3	—	
97·5 % C_{27}+2·5 % C_{28}	52·5–53°	59·0–59·2°	58·8°	—	B 56	36·3	—	
95 % C_{27}+5 % C_{28}	52·5–53°	59·1–59·3°	58·9°	50°	B 57	36·45	—	
90 % C_{27}+10 % C_{28}	52·5–53°	59·2–59·5°	58·9°	50°	B 58	36·55	—	
99 % C_{28}+1 % C_{27}	56·8°	61·2–61·4°	61·1°	53·4°	B 66	—	33·2	
97·5 % C_{28}+2·5 % C_{27}	56·4°	61·2–61·4°	61·0°	53·3°	B 67	—	33·3	
95 % C_{28}+5 % C_{27}	55·6°	61·2–61·4°	61·0°	53·2°	B 68	—	33·2	
90 % C_{28}+5 % C_{27}+5 % C_{29}	55·2°	61·3–61·5°	61·2°	—	B 69	37·7	—	
90 % C_{28}+5 % C_{26}+5 % C_{30}	54–54·4°	61·3–61·5°	61·2°	—	B 70	38·0	—	
97·5 % C_{32}+2·5 % C_{31}	64°	69·4–69·6°	68·8°	63·4°	B 98	—	42·5	
C_{27}+C_{28}+C_{29}	53·4°	61·0–61·4°	60·8°	—	B 54	38·25	—	
C_{28}+C_{29}+C_{30}	50–51°	61·6–61·8°	61·3°	—	B 71	—	—	
C_{26}+C_{27}+C_{28}+C_{29}+C_{30}	44°	61–61·5°	60·7°	—	B 79	—	—	
C_{27}+C_{29}+C_{31}	52·3°	63·5–63·6°	62·8–63°	—	C 86	40·2 37·75	—	Approximate to the spacings of the constituents
C_{29}+C_{30}+C_{31}	58–59°	65·6–65·7°	65·4°	—	C 84	40·8	—	
C_{31}+C_{35}	55°	70·7–71·2°	70·3–70·5°	—	C 82/3	46·25 43·0	—	
C_{34}+C_{35}	69°	73·6–73·9°	73·3°	—	C 89	46·05	—	

able change in *C* spacing until the *A* form appears, spacing variations then follow the course described for two odd number paraffins.

A study of Plate VIII, Fig. 1, reveals marked differences in the intensity decrease with increasing order as the mixtures approach equimolar proportions. Little radiation is reflected beyond the fourth order, and the four orders that

do appear stand out much more sharply than in the plates of the more nearly pure materials. To make such a comparison fairly it is of course necessary to take the whole range of photographs under identical conditions. An experienced eye can tell from a glance at a photograph whether or not a given specimen is approaching purity, though in the case of odd number paraffins which have no *C* form, it is easy to overestimate the degree of purity attained.



× =X-ray measurements with mixed paraffins

Figs. 4, 5, 6 and 7.

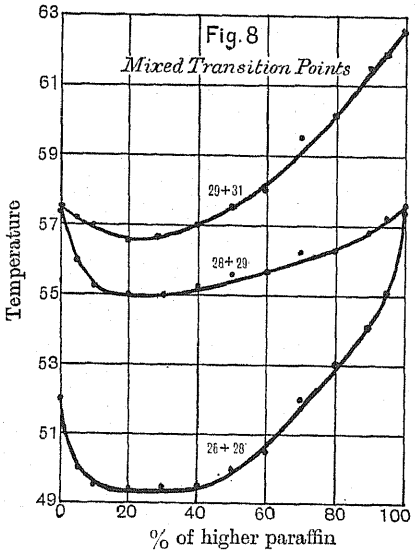
A few complicated mixtures have been examined, but X-ray data lead to no important generalisations. It will however be observed that in equimolar binary mixtures of chains that differ by 4 or more carbon atoms, distorted crystals of both constituents separate, though in the case of octacosane and dotriacontane a melted layer gave only one spacing. In this connection it may be noted that the *A* spacing of octacosane is 37 and the *C* value of dotriacontane 37·8. The mixed crystal from the melt may consist of tilted 32 and upright 28 molecules though the value found, 40 Å., is high.

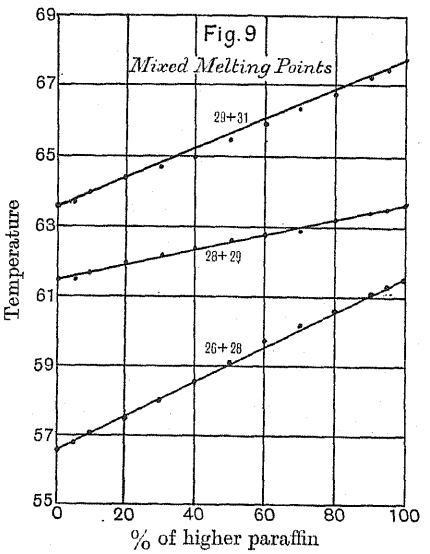
The melting- and transition-points of mixtures of paraffins.

The melting-, setting- and transition-temperatures of a comprehensive series of mixed paraffins are given in Tables II–V. It is proposed to summarise here only those observations which will aid an observer in judging the purity of a given sample of a paraffin.

Melting-point. The melting-points of mixtures of two paraffins given in Tables II–IV lie in each case on straight lines, see Fig. 9. There is no depression similar to that observed with the fatty acids, alcohols or ketones, so that (for instance) equimolar $C_{26}H_{54}+C_{28}H_{58}$ melts at the same temperature as $C_{27}H_{56}$ (59·1°) and equimolar $C_{29}H_{60}+C_{31}H_{64}$ (65·6°) the same as $C_{30}H_{62}$ (65·7°). In the

case of equimolar mixtures of three paraffins, the melting-points are still quite sharp and correspond with the mean molecular weight. In the case of equimolar mixtures of four or more paraffins the melting-points are indistinct, but are in the region corresponding with the mean molecular weight. To give a concrete example the following paraffins and mixtures all melt at approximately the same temperature (61·4–61·6°): $C_{28}H_{58}$, equimolar $C_{27}H_{56}+C_{29}H_{60}$, $C_{28}H_{58}+5\ \%\ C_{27}H_{56}+5\ \%\ C_{29}H_{60}$, $C_{28}H_{58}+5\ \%\ C_{26}H_{54}+5\ \%\ C_{30}H_{62}$, equimolar $C_{26}H_{54}+C_{28}H_{58}+C_{30}H_{62}$, equimolar $C_{26}H_{54}+C_{27}H_{56}+C_{28}H_{58}+C_{29}H_{60}+C_{30}H_{62}$. It is obvious from these results that the purity of a paraffin cannot be judged by a comparison of melting-point, or by mixed melting-point with a sample of a paraffin of known purity.





Setting-points. In the case of equimolar mixtures of two or three paraffins whose carbon content does not differ by more than two or three carbon atoms, the setting-points are quite sharp and are not appreciably depressed. In the case, however, of a mixture of, say, two paraffins differing by three or more carbon atoms it will be found that the melting-point extends over a somewhat broad range, 0·5–0·7°, and that the upper limit of the setting-point may be higher than the lower limit of the melting-point. This is due to the separation of quite large crystal masses containing an excess of the higher melting constituent. The fact that the setting-point is very close to the melting-point, therefore, is not necessarily a guarantee that the paraffin is pure. Equimolar mixtures containing more than three paraffins do not set crystalline; instead a white dense mist slowly separates from the bottom of the tube upwards.

Transition-points. As we believe that the transition-point alone can serve as a satisfactory guide throughout the separation of a mixture of naturally

occurring paraffins, these call for more extended comment. In the case of mixtures of neighbouring paraffins, for example $C_{28}H_{58}$ and $C_{29}H_{60}$ given in Table II, the transition-temperatures on heating and cooling are quite sharp throughout the whole range. If the two paraffins, however, differ by two carbon atoms, then the transition-points on both heating and cooling between the limits of 30 and 70 % of one constituent are not sharp and occur gradually over a range that may be 0·5–1°. In the case of small amounts of impurities, such as 1–5 % of another paraffin, the sharpness of the transition-point is not affected. If however any of these paraffins are melted side by side in the bath with the pure paraffin there is always a recognisable difference in the temperature of transition on heating and especially on cooling.

A careful observation of the transition-point in fact will detect 1 % impurity in a paraffin. It is obvious that the exact temperature at which this transition occurs will depend a little on the rate of heating or cooling of the bath and therefore to make full use of this transition-point it is necessary to have a sample of chemically pure paraffin for comparison.

We have carefully compared the melting-points and transition-points of pure paraffins with those containing other paraffins in the small proportion of 1–5 % as given in Table V. The melting-points of the latter in all cases are raised or lowered according as to whether the admixed paraffin has a longer or shorter chain. When such samples are melted side by side the difference in melting-point is observable though it may be too small to be stated. However, the values obtained for transition-points, especially on cooling, differ appreciably and are never less than 0·2°.

In general our observations suggest that the even number paraffins containing 1–5 % of a neighbouring paraffin show considerably more depression in the transition-point on heating than do the odd number paraffins (Fig. 8).

In the case of approximately equimolar mixtures of two paraffins separated by three or more carbon atoms, the transition-point is not well defined. The contents of the melting-point tube viewed at room temperature show very little crystalline structure. A glassy state is already apparent, and on heating there is no definite first transition. The second transition is however very marked and occurs at 1–1·5° instead of 0·3–0·5° below the melting-point. On cooling the first transition to the glass takes place normally, and this form persists down to room temperature with, perhaps, the appearance of a few cleavages, showing partial reversion to the more usual crystalline form. As will be emphasised later, this marked difference in behaviour is of great practical use.

In the case of equimolar mixtures of three or four paraffins the transition-temperature is very indistinct and occurs at that of the lowest member in the mixture. The transition-point, therefore, can be 10–15° below the actual point of fusion. The one sample of an equimolar mixture of five paraffins that was made, $C_{26}H_{54}$ to $C_{30}H_{62}$ inclusive, showed no appreciable transition-points at all.

GENERAL DISCUSSION.

It will be seen from the experimental data given above that if a paraffin is to be considered pure it must have not only the correct melting-point, but also the correct transition-point. Its spacing will be correct to 0·2 % and the picture will show 8 or more orders. It will also give good microcrystals which display a sharp rhombic cleavage, the angles of which will differ according as to whether the paraffin has an odd or even number of carbon atoms.

In biochemical investigations it is improbable that paraffins of this high degree of purity will be often encountered. Recognising this fact we have, in this research, aimed not only at the establishment of these absolute criteria of purity, but also the collection of evidence which will enable us to suggest the composition of a mixture from which we are unable to separate a chemically pure paraffin. In the following summary we give a number of conclusions which are already aiding us in our investigations of plant waxes. In passing it may be mentioned that mixed paraffins can be readily separated into fractions having different melting-points by means of light petroleum, but that the preparation of a single paraffin of recognisable degree of purity from a mixture is always very laborious, and according to our experience, often impossible.

(1) If a paraffin gives the correct melting-point, and also the correct transition-temperature on both heating and cooling, then it contains less than 1 % of impurity. In such cases confirmation by X-ray analysis is unnecessary.

(2) X-ray analysis alone, without due regard to melting- and transition-points, is insufficient to identify a paraffin. Reference to Table III will show that $C_{31}H_{64}$ and a mixture of 20 % $C_{29}H_{60}$ + 80 % $C_{31}H_{64}$ both have the same spacing (41·5 Å.).

(3) The melting-point curve of a mixture of two paraffins which do not differ by more than 2 carbon atoms is a straight line (Fig. 9). This means that the melting-point of a mixture is determined by its mean molecular weight, and that no depression is exhibited. Within similar limits as to carbon atoms the same is true for mixtures of three paraffins. In respect to melting-point therefore the mixed paraffins are sharply differentiated from the alcohols and acids, which show in most cases very considerable depression. This finding has a very important practical application. In separating the constituent paraffins of a mixture it follows that of any two fractions obtained the one with the higher melting-point will have the higher mean molecular weight. This, of course, is not necessarily so with mixtures of acids or alcohols, in which both the higher and lower acids or alcohols may have melting-points higher than that of the respective mixtures. Francis, Piper and Malkin [1930, p. 227] came to the conclusion that it was extremely difficult, and in some cases impossible, to effect the separation of mixed acids by fractional crystallisation, and it is probable that this also applies to alcohols.

Further we have collected a very large number of observations on the crystallisation of mixed paraffins, and since their layer structure is simpler

than those of the acids and alcohols (which contain the length of two molecules between the 001 planes) the interpretation of the X-ray photographs, at this stage, is more straightforward. For this reason we are investigating the composition of the mixed primary alcohols which occur in plant waxes by fractionation of the derived paraffins, in preference to the fractionation of the alcohols themselves or the derived acids as done by previous workers.

(4) It follows from the data given in the above section that a mixture of two paraffins can give the same melting-point as a pure paraffin, but the transition-point will be lower. When such a mixture is melted with this pure paraffin the melting-point will be unchanged, but the transition-point will be either that of the mixture or lower. Therefore an observation confined to the mixed melt—which is the usual laboratory practice—is, by itself, quite insufficient to warrant any conclusion as to purity. The transition-temperature gives this information at once.

(5) If a mixture has a recognisable transition-point, a sharp melting-point, and sets crystalline when the temperature on cooling falls about 0·5°, it is safe to assume that there are not more than three components present in recognisable amounts. If the material melts over an extended range (0·5–1°), and if on cooling crystals come out at a temperature on or above the lower of these limits, then the mixture is probably of two paraffins only whose carbon contents differ by three or more carbon atoms.

(6) The short spacing of an even number paraffin will come up although the sample contains 2·5 % of a longer or up to 10 % of a shorter paraffin. A relatively impure sample of an even number paraffin will therefore show a short spacing which is quite specific and is a positive identification. Unfortunately no such simple means are available for the positive identification of an odd number paraffin of this degree of purity.

(7) In a series of fractions obtained from a mixed paraffin which does not appear to contain more than three components our experience suggests that if the transition-temperatures are some 8–10° below the melting-points, components are even number paraffins, and if 6–8° they are odd. This conclusion must be accepted with caution, as the transition-temperatures are susceptible to very small amounts of impurities.

(8) When attempting to decide upon the constituents of a naturally occurring paraffin in which the maximum degree of separation has been obtained, we first measure the spacing. We are then able to select one or more binary synthetic mixtures which have the same spacing. Of these synthetic mixtures we choose the one in which both the M.P. and transition-point are in best accord with those of the mixture under examination. It is unusual to find a perfect fit, but quite common to find reasonable agreement between the spacings and M.P. Transition-points are considerably depressed by the addition of a small amount of impurity, and it is usual to find the transition-point, when not agreeing with a synthetic mixture of corresponding M.P. and spacing, to be low. In such cases we consider the main constituents of the mixture under

examination to be those of the best agreeing binary mixture with a small (about 5 %) impurity whose constitution we are unable definitely to state. Since we are concerned with establishing the actual presence in bulk of the main constituents rather than in estimation of the exact proportion in which they occur, it is our practice to find the spacings of mixtures which we have not actually prepared from graphs shown to correspond with those of similar mixtures whose spacings have been measured. Thus the spacing curve for $C_{28}H_{58}$ and $C_{30}H_{62}$ is taken to be identical with that for $C_{26}H_{54}$ and $C_{28}H_{58}$, but the scale of ordinates is raised to lie between the appropriate spacings of $C_{28}H_{58}$ and $C_{30}H_{62}$. Similar estimations have been made for transition-points. In each case we have checked this assumption by making one or two appropriate mixtures.

It will be seen from the above recommendations that our experiments, which have been guided throughout by X-ray analysis, bring out quite clearly the importance of the transition-point. In our investigations of plant waxes we are, in fact, now able to dispense entirely with the laborious X-ray analysis in all cases except (*a*) for positive identification, especially with even number paraffins, (*b*) as a guide to the composition of mixtures from which we are unable to obtain fractions of the requisite purity by fractional crystallisation. In the two papers which follow we describe the fractionations of plant waxes which were carried out at the same time as the work described in the present paper. In these cases X-ray analysis was freely used and it will be seen that the final results give, in several instances, practical confirmation of the recommendations given above.

The position of side chains.

Two important papers by Shearer [1925] and Savile and Shearer [1925] have shown how the position of a side-group in a straight chain may be determined by X-rays. We have used this method for identifying ketones and secondary alcohols.

The method may be understood from an inspection of Plate VIII, Fig. 3, which shows the spacings of a series of nonacosanones. All the chain lengths are the same, and the spacings, within 0·25 %, agree with those for nonacosane. The intensities of the lines vary in a marked manner, displaying a periodic intensity variation superimposed on the normal decrease with increasing order. A group at $\frac{1}{3}$ of the chain length gives weak 3rd, 6th, 9th, *etc.*, orders; half-way along the chain it causes the even orders 2, 4, 6, *etc.*, to be very faint. We find that the secondary alcohols have intensity fluctuations of an identical nature, but that the spacings are very much shorter, showing that the molecule is inclined. Also they have both a high and a low temperature form. The spacings give the chain length, and the intensity fluctuations decide the position of the group within about 2 carbon atoms. It must be added that Shearer has pointed out that when the side group is on the last or the penultimate carbon atom the

Table VI. *Melting-points and crystal spacings of ketones and secondary alcohols.*

Abbreviated formula of compound	M.P.	Series number	Crystal spacing in Å.		Remarks
$C_{14}-CO-C_{14}$	80·8°	C 1	38·65		The constant spacing but variable intensity distribution are shown in Plate VIII, Fig. 3
$C_{13}-CO-C_{15}$	74·7°	C 47	38·9		
$C_{11}-CO-C_{17}$	74·7°	C 48	38·9		
C_9-CO-C_{19}	75·0°	C 49	38·9		
C_8-CO-C_{20}	74·4°	—	38·8		
			High temp.	Low temp.	
$C_{11}-CHOH-C_{11}$	75·2°	C 6	—	31·5	
$C_{13}-CHOH-C_{13}$	80·5°	—	34·75	36·6	
$C_{14}-CHOH-C_{14}$	84·0°	—	—	37·15	Spacing lower than for the corresponding ketone
$C_{13}-CHOH-C_{15}$	79·5°	—	—	—	Spacing not observed
$C_{11}-CHOH-C_{17}$	74·7°	—	—	36·9	
$C_9-CHOH-C_{19}$	75·0°	—	—	—	
d-$C_9-CHOH-C_{19}$	82·2°	C 75	—	38·65	Note the high value of this spacing. It corresponds to the A spacing of n-nonacosane
$C_8-CHOH-C_{20}$	75·8°	C 55	—	37·15	
$C_{15}-CHOH-C_{15}$	84·7°	—	39·4	41·25	

Table VII. *Melting-points and crystal spacings of primary alcohols.* Malkin [1930].

Number of C atoms	M.P. (Malkin)	M.P. (A.C.C.)	Crystal spacing in Å. High temperature	Crystal spacing in Å. Low temperature
16	49·0°	—	44·9	37·4
18	59·0°	—	50·2	41·35
19	62·0°	—	52·8	—
20	—	65–65·5°	—	46·2
21	68·5°	—	56·9	57·4
22	72·0°	—	—	49·95
24	76·5–77°	75·6–76°	—	54·0
25	78·5°	77·7–78°	—	68·5

The spacing and M.P. of normal eicosanol are new measurements.

spacing is doubled, owing to crystallisation occurring with 2 carbon chains between the 001 planes like end to like end.

The melting-points and crystal spacings of the secondary alcohols and ketones which appear to us of biochemical importance are given in Table VI. Equimolar mixtures in all cases show a depression of many degrees. We have to thank Dr Malkin for permission to reproduce his data for the higher primary alcohols given in Table VII.

Summary.

Highly pure paraffins from $C_{26}H_{54}$ to $C_{36}H_{74}$ and certain long-chain ketones and secondary alcohols of possible biochemical importance have been synthesised and their crystal spacings determined.

If the melting-point of a pure paraffin is determined in the way described it is possible to determine accurately two transition-temperatures at which a visible change of structure occurs. The lowest of these is markedly affected by impurities and is shown to be of great practical importance.

The melting-points, transition-points and crystal spacings of a comprehensive series of mixed paraffins have been determined.

From the data thus obtained it has been possible to draw up a series of recommendations which will aid in the fractionation and identification of the mixed paraffins which occur in nature, or which can be obtained from the mixed primary alcohols which occur in nature.

Brief reference is made to the work of Shearer [1925] showing how the position of a side group in a straight chain can be determined by X-rays.

In conclusion we should like to thank Prof. Garner and Dr Malkin for supplying us with samples of highly pure paraffins and alcohols, and the Imperial Chemical Industries, Ltd., for a grant towards the expenses of this research.

REFERENCES.

Backmann and Clark (1927). *J. Amer. Chem. Soc.* **49**, 2089.
Brooks (1922). The non-benzenoid hydrocarbons. (New York.)
Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Drew and Porter (1928). *J. Soc. Chem. Ind.* **47**, 17.
Francis, Piper and Malkin (1930). *Proc. Roy. Soc. Lond.* A **128**, 214.
Garner, van Bibber and King (1931). *J. Chem. Soc.* 1533.
Gascard (1921). *Ann. Chim.* (9), **15**, 332.
Gluud (1919). *Ber. deutsch. chem. Ges.* **52**, 1051.
Grün, Ulbrich and Krczil (1926). *Z. angew. Chem.* **39**, 421.
Hildebrand and Wachter (1929). *J. Amer. Chem. Soc.* **51**, 2487.
Kraft (1882). *Ber. deutsch. chem. Ges.* **15**, 1714.
Levene, West and Van der Scheer (1915). *J. Biol. Chem.* **20**, 521.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Müller and Savile (1925). *J. Chem. Soc.* **127**, 602.
—— —— (1928). *Proc. Roy. Soc. Lond.* A **120**, 437.
—— —— (1930). *Proc. Roy. Soc. Lond.* A **127**, 417.
Peterson (1906). *Z. Electrochem.* **12**, 141.
Piper, Brown and Dyment (1925). *J. Chem. Soc.* **127**, 2194.
—— Malkin and Austin (1926). *J. Chem. Soc.* 2310.
—— and Malkin (1930). *Nature*, **126**, 278.
Pregl (1924). Quantitative organic analysis. Trans. Fyleman. (London.)
Robinson and Robinson (1925). *J. Chem. Soc.* **127**, 2194.
Savile and Shearer (1925). *J. Chem. Soc.* **127**, 592.
Shearer (1925). *Proc. Roy. Soc. Lond.* A **108**, 655.

CCXXVI. THE WAX CONSTITUENTS OF THE APPLE CUTICLE.

By ALBERT CHARLES CHIBNALL, STEPHEN HARVEY PIPER, ALFRED POLLARD, JAMES ANDREW BUCHAN SMITH and ERNEST FRANK WILLIAMS.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington, and the Wills Physical Laboratory, University of Bristol.

(*Received October 31st, 1931.*)

Introduction.

In the introduction to the preceding paper dealing with the synthesis and crystal spacings of certain higher paraffins, alcohols and ketones, it was pointed out that the object of the work was to enable a more thorough investigation of the chemical constitution of various plant waxes, especially leaf waxes, to be made than had hitherto been possible. The leaf waxes to which it was hoped particular attention could be given were those derived from various forage grasses, and preliminary investigations showed that these differed from that found by Channon and Chibnall [1929] in the cabbage leaf in that they contained large amounts of alcohols instead of a paraffin and ketone and were therefore not immediately amenable to the separation used by these authors. As a preliminary to work on the grasses it was necessary to devote some time to experiments, the aim of which was to separate fairly completely the following classes of compounds, *viz.* paraffins, ketones, primary alcohols and secondary alcohols, and because a survey of the literature suggested that these might all occur in the wax obtained from apple peel, that material, which can readily be obtained in quantity, was used.

Before describing the actual scheme of separation it may perhaps be as well to state briefly the results of previous workers who have investigated this apple peel wax. Power and Chesnut [1920] isolated a substance, m.p. 63°, which appeared to be a hydrocarbon and which was identified from its melting-point as *n*-triacontane $C_{30}H_{62}$. A more comprehensive investigation was made by Sando [1923], who extracted the peel with ether and obtained two groups of substances, one insoluble in light petroleum with which we are not now concerned, and the other soluble in light petroleum, from which he was able to obtain, by intensive fractional crystallisation, the hydrocarbon previously identified by Power and Chesnut and an alcohol melting at 81·5 to 82°. This alcohol gave an acetate melting at 44–46° and was therefore thought to be different from ceryl alcohol, which gives an acetate, m.p. 64–65°. 14-Heptacosanol, previously synthesised by Kipping [1893] melted at 80·5–81·5°

and gave an acetate melting at 45–45·5°. On a similarity of melting-point and without any further evidence at all, Sando identified his apple alcohol with this secondary alcohol, a finding which has been accepted without question by Rivière and Pichard [1924]. Sando's results suggested that other products besides this alcohol and hydrocarbon were probably present in his apple material. Previous work carried out by Power and also in this laboratory suggested that these other products might be primary alcohols or ketones, and the wax, therefore, seemed to be an excellent raw material with which to experiment on methods to separate these four products.

A METHOD FOR THE SEPARATION OF THE COMPONENTS OF PLANT WAXES.

It has long been known that primary alcohols, such as ceryl and myricyl alcohol, form hydrogen phthalates which give sodium salts insoluble in water, and this property has been utilised by a number of workers, notably by Power and his colleagues, for the isolation of such alcohols from plant extracts and especially from essential oils. With a view to finding out the applicability of this reaction to the separation of primary alcohols from material that may contain paraffins, ketones and perhaps secondary alcohols, experiments were made to find out the optimum conditions for the conversion of the alcohol into its hydrogen phthalate.

Using ceryl alcohol (M.P. 79·7°) obtained from Chinese wax it was found that heating with an excess of phthalic anhydride with or without pyridine at 120° for 18 hours gave the half ester in almost theoretical yield. On shaking the ethereal solution with aqueous sodium carbonate the insoluble sodium salt was precipitated and could be separated by centrifuging. The alcohol could be recovered from this sodium salt or from the half ester in good yield by saponification with alkali. When a secondary alcohol, however, such as 14-heptacosanol, was submitted to similar treatment it was found that the hydrogen phthalate when shaken in ethereal solution with aqueous sodium carbonate gave no precipitate of insoluble sodium salt. The sodium salt, in fact, was found to be extremely soluble in both ether and alcohol and insoluble in water. This at once suggested a possible method of separating a secondary carbinol not only from primary alcohols but also from paraffins and ketones, and in practice it has been found to work extremely well.

The crude plant wax is first saponified to break down any true wax esters and the unsaponifiable material removed in the usual way. This unsaponifiable material is then heated for 18 hours with phthalic anhydride to convert all the alcohols present into hydrogen phthalates. Excess of phthalic anhydride is then removed and the mixed phthalates, together with any unchanged material, dissolved in ether. The ethereal solution is then gently shaken with a slightly warm aqueous solution of sodium carbonate. The sodium salts of the primary phthalates are precipitated and can be quantitatively separated by appropriate treatment. The ethereal solution which now contains the sodium salts of the secondary phthalates and perhaps ketones and paraffins is evaporated

to dryness and taken up in a relatively small volume of boiling alcohol. On cooling, the paraffins and ketones crystallise out quantitatively leaving in the mother-liquor the very soluble sodium salts of the secondary alcohol esters. The mixture of paraffins and ketones can then be separated by the method used by Channon and Chibnall [1929] in which the ketone is converted into its oxime and advantage is taken of the greater solubility of this oxime in solvents such as light petroleum and mixed ether-acetone. It will be seen that this relatively simple procedure separates at once the primary alcohols, the secondary alcohols, ketones and paraffins into four distinct fractions, each of which can be readily purified. In practice certain modifications have been found essential. For instance, a second treatment with phthalic anhydride for 18 hours is always necessary to ensure that all the alcohols have been converted into their hydrogen phthalates. Also recent experience has shown that it is advantageous to remove hydrocarbons—which are fairly readily soluble in cold light petroleum—before treatment with phthalic anhydride. The method and its modifications will be dealt with in greater detail in the practical part of this and succeeding papers.

The method of separation applied to apple peel wax.

Applied to the unsaponifiable portion of apple peel waxes (50 g.) this method of separation yielded without any difficulty a secondary alcohol, a mixture of primary alcohols and a mixture of paraffins but no ketones.

The secondary alcohol. The alcohol recovered after saponification of the ether- and alcohol-soluble sodium salts on purification melted at 81·9–82·2° and was obviously the product previously isolated by Sando [1923], for it gave an acetate melting at 44·5–45°. A mixed melt however with 14-heptacosanol was depressed to 70–71°, showing conclusively that Sando's guess was wrong. One of the reasons which prompted us initially to investigate apple peel wax was the belief that this alcohol might be 15-nonacosanol, the secondary alcohol corresponding with the ketone, 15-nonacosanone and the paraffin, *n*-nonacosane, isolated by Channon and Chibnall [1929] from the cabbage. Synthetic 15-nonacosanol, however, melted somewhat higher, 83·8–84°, and a mixed melt was depressed to 78·5–79·5°, showing that the alcohols were not identical. The carbinol had a spacing of 38·65 Å., indicating a straight chain of 29 carbon atoms. Furthermore, the intensity of the different orders of reflection showed that the polar group must be about one-third of the distance along the chain from one end because the third and sixth orders were missing. X-ray analysis, therefore, suggested that the alcohol must be either 9-nonacosanol or 10-nonacosanol. To verify this supposition it was necessary to synthesise both these alcohols *via* the corresponding ketones. Meanwhile, in order to meet the synthesis, the apple alcohol was oxidised by means of chromium trioxide in glacial acetic acid to the corresponding ketone. The apple ketone, M.P. 74·7–74·9°, showed no depression when mixed with synthetic 10-nonacosanone, M.P. 74·5–75°, and a depression of 5° when mixed with synthetic 9-nonacosa-

none, M.P. 73·6–74·3°. Furthermore, the crystal spacings (Series Nos. C 35, C 49) of the derived apple ketone and of the synthetic 10-nonacosanone were identical (38·9 Å.), both, as before, having the third and sixth orders missing. The identity of the ketone derived from the apple alcohol was therefore definitely established. On reducing the synthetic 10-nonacosanone to the corresponding carbinol, 10-nonacosanol, however, it was found to melt at 75–75·2°, *i.e.* 7° lower than the apple alcohol. A mixed M.P. (78–79°) was about midway between the melting-points of the two substances, whereas a mixed melt of the apple alcohol with synthetic 9-nonacosanol (M.P. 75·5–75·8°) was depressed to 70°. Clearly the apple alcohol was not identical with the synthetic 10-nonacosanol, although no doubt closely allied to it. This finding at once drew our attention to the fact that if the apple alcohol had the constitution which its derived ketone suggested, it would possess an asymmetric carbon atom and therefore would be optically active, whereas the alcohol obtained by reduction of the ketone would of course be inactive. Dr Hirst of the University of Birmingham kindly undertook to observe the rotation of the apple alcohol, and he reported as follows: "The substance was examined in solution in chloroform (*c*, 1 %). The rotation observed in a 4 dm. tube was certainly less than the experimental error due to the instrument (± 0·01°). Photographic examination over the range λ 7000–λ 3800 in a 2 dm. tube showed that throughout this range there was no observable rotation (limits of experimental error here were ± 0·02°). It is therefore most improbable that the substance is optically active either in chloroform or in any other solvent." There remained the possibility that the length of the two paraffin side-chains was so great that optical activity might be reduced below measurable limits. This hypothesis was confirmed when the ketone obtained from the apple alcohol by oxidation was reduced. The regenerated alcohol melted at 74·5–74·9° and gave no depression in M.P. with the synthetic product, which melted at the same temperature. Attempts to racemise the natural product by heating gave no change in melting-point. At a later stage of the research we were able to prepare some 4 g. of the hydrogen phthalate. A 20 % solution in chloroform gave a small but definite rotation $[\alpha]_{5461}^{17\cdot5} = +0\cdot62°$. The apple secondary alcohol is therefore undoubtedly *d*-10-nonacosanol and it belongs to a small group of optically active compounds, of which mannitol is a well known example, exhibiting no appreciable rotation. It is possibly identical with the alcohol, M.P. 82·5°, isolated by Kawamura [1928] from the fruit of *Ginkgo biloba* which gave an acetate M.P. 43–43·5° and a ketone M.P. 74°.

Primary alcohols. The ether-insoluble sodium salts of the primary phthalates were saponified and yielded, after purification by crystallisation, 4·1 g. of primary carbinol. This material melted at 81° and when submitted to an intensive fractional crystallisation yielded fractions differing in M.P. by 1 and 2°. Investigations of these fractions soon convinced us that we were dealing with a mixture of primary alcohols of the type usually referred to as ceryl alcohol, the constitution of which will be discussed in greater detail in the

following paper. An upper fraction, M.P. 81·7–82·2°, gave an analysis corresponding closely with *n*-octacosanol. (Found: C, 81·8; H, 14·1. $C_{28}H_{58}O$ requires C, 81·8; H, 14·2 %.) The crystal spacing (Series No. C. 56) was 77·6 Å. which suggests that the product, according to the data of Malkin [1930] was $C_{29}H_{60}O$. On reduction *via* the iodide it gave a paraffin M.P. 62·2–62·8°. The crystal spacing (Series No. C. 72) was 38·4 Å. with only 5 orders showing, and the paraffin was clearly a mixture. A lower fraction, M.P. 80·2–80·7°, gave a spacing (Series No. C. 33) of 76·1 Å. and a derived paraffin which melted at 60–60·2°. The crystal spacing (Series No. C. 46) of this was 37·4 Å. with only three orders showing and again the material was definitely a mixture. All the fractions of this primary alcohol therefore were united and reduced *via* the iodide to the corresponding paraffin, M.P. 61·0–61·5°, which was submitted to an intense fractionation by means of light petroleum. This was found to be very laborious. After about 100 fractions had been dealt with the upper and lower limits of M.P. were 62·8° and 59·5°, but the main bulk of material still melted around 61° and 62°. The highest and lowest fractions were again treated with sulphuric acid. At this stage of the resolution we found that all the fractions showed certain characteristics. (1) The M.P. was sharp, and about 0·2–0·3° above the S.P. (2) On setting, the molten material crystallised out in fine needles. (3) The transition changes were well marked, but the temperature range in some cases was 1–2°. This convinced us that we were dealing with a mixture containing not more than 3 components in recognisable amounts, but the resolution was not sufficiently complete for X-ray analysis to be of any use. A fraction (Series No. B. 80) of M.P. 61·4–61·6°, corresponding to $C_{28}H_{58}$, gave a spacing 38·3 Å. with a fair number of orders, a melted layer however gave a poor picture and a spacing of 37·8 Å. This distortion of a spacing on melting is characteristic of a mixture of at least three paraffins, and showed that it was necessary to carry the fractionation still further. After another 150 fractions had been dealt with we reached a stage when further fractionation by this means seemed impossible. We had then 22 samples. All the fractions set on cooling in large crystal masses, but in none was the transition-temperature as high as that of a corresponding pure paraffin. We drew the conclusion that the fractionation had separated the three components to such an extent that each sample now contained only two of these in recognisable amounts. Ten representative samples were then X-rayed, and as was expected, the picture obtained was sufficiently good to enable a comprehensive analysis of the mixtures to be obtained.

A review of the data in Table I shows that it is possible to account for all the melting- and transition-points and the spacings from suitably chosen mixtures of $C_{30}H_{62}$, $C_{28}H_{58}$ and $C_{26}H_{54}$. On this assumption the bulk of the material in B. 115 is the middle paraffin with about equal amounts of the higher- and lower-melting constituents as impurities. In B. 110–B. 114 we have mainly $C_{30}H_{62}$, $C_{28}H_{58}$; in B. 116–B. 119 $C_{28}H_{58}$, $C_{26}H_{54}$. The transition-temperatures are nearly all slightly lower than those of the suggested binary

Table I. *Paraffin fractions obtained from the mixed primary alcohols.*

Series No.	M.P.	S.P.	Transition-point on heating	Spacing Å.	Synthetic mixture: Series No.	Synthetic mixture: Composition	M.P.	S.P.	Transition-point on heating	Spacing Å.	Remarks
B. 110	63·2–63·6°	63·2°	54·5°	39·25	Computed data	60 % $C_{28}H_{58}$ 40 % $C_{30}H_{62}$	63·2°	—	54·8	39·25	The photograph of B. 110 is definitely that of a paraffin with 30–40 % contamination.
					C. 97	95 % $C_{29}H_{60}$ 5 % $C_{31}H_{64}$	63·6°	63·1°	57·2°	39·15	C. 97 gives very good pictures.
					Computed data	90 % $C_{29}H_{60}$ 10 % $C_{30}H_{62}$	64°	—	55·2°	39·25	
B. 112	63·1–63·3°	63·1°	54–55°	38·85	Computed data	65 % $C_{28}H_{58}$ 35 % $C_{30}H_{62}$	63°	—	55°	39	
						$C_{29}H_{60}$	63·4–63·6°	—	57·3°	38·68	A very small % of $C_{30}H_{62}$ or $C_{31}H_{64}$ in the $C_{29}H_{60}$ would give the correct spacing. The photograph however is definitely that of a paraffin with 30–40 % of contamination.
B. 113	62·6–62·8°	62·4°	53·7–54·2°	38·55	Computed data	75 % $C_{28}H_{58}$ 25 % $C_{30}H_{62}$	62·5°	—	54·8°	38·5	
					B. 89	50 % $C_{29}H_{60}$ 50 % $C_{28}H_{58}$	62·3–62·6°	62·3°	55·6°	38·5	
					Computed data	35 % $C_{27}H_{56}$ 65 % $C_{29}H_{60}$	62°	—	54°	38·5	
					B. 122	34 % $C_{26}H_{54}$ 66 % $C_{29}H_{60}$	60·7–61°	60·7°	50–52°	38·6	
B. 114	62·2–62·6°	62°	53·2–53·7°	38·55	B. 89	50 % $C_{29}H_{60}$ 50 % $C_{28}H_{58}$	62·3–62·6°	62·3°	55·6°	38·5	
					Computed data	75 % $C_{28}H_{58}$ 25 % $C_{30}H_{62}$	62·5°	—	54·8°	38·5	
					B. 125	74 % $C_{28}H_{58}$ 24 % $C_{30}H_{62}$ 2 % $C_{26}H_{54}$	62·3–62·5°	62·0°	53–53·5°	38·5	

Series No.	M.P.	S.P.	Transition-point on heating	Spacing Å.	Synthetic mixture: Series No.	Synthetic mixture: Composition	M.P.	S.P.	Transition-point on heating	Spacing Å.	Remarks
B. 115	61·2–61·5°	61·2°	52–52·7°	38	Computed data	85 % $C_{28}H_{58}$ 15 % $C_{30}H_{62}$	62·1°	—	54·8°	38	
					C. 114	$C_{28}H_{58}$	61·4–61·5°	61·1°	57–57·4°	37 33·25	There was no appearance of the shorter spacing in B. 115.
					B. 123	50 % $C_{27}H_{56}$ 50 % $C_{29}H_{60}$	61·1–61·3°	60·9°	52–52·5°	38·2	
					B. 70	**90 % $C_{28}H_{58}$** 5 % $C_{26}H_{54}$ 5 % $C_{30}H_{62}$	**61·3–61·5°**	**61·2°**	**54–54·4°**	**38**	Appears to be the most probable constitution.
					B. 69	90 % $C_{28}H_{58}$ 5 % $C_{27}H_{56}$ 5 % $C_{29}H_{60}$	61·3–61·5°	61·2°	55°	37·7	
B. 116	59·9–60·1°	59·8°	50–51°	37·65	B. 47	**70 % $C_{28}H_{58}$** **30 % $C_{26}H_{54}$**	**60·1–60·2°**	**60°**	**52°**	**37·7**	
B. 117	58·0–58·4°	58°	47·2–47·4°	36·65	B. 46	**60 % $C_{26}H_{54}$** **40 % $C_{28}H_{58}$**	**58·6°**	**58·2°**	**49·5°**	**36·8**	
					B. 58	90 % $C_{27}H_{54}$ 10 % $C_{28}H_{56}$	59·2–59·5°	58·9°	52·5–53°	36·6	
B. 118	57·2–57·6°	57·1°	46–46·5°	36·24	B. 44	**70 % $C_{26}H_{54}$** **30 % $C_{28}H_{58}$**	**58°**	**57·5°**	**49·5°**	**36·2**	
B. 119	56·8–57°	56·6°	46·5–47°	35·55	B. 42	90 % $C_{26}H_{54}$ 10 % $C_{28}H_{58}$	57°	56·3°	49·5°	35·25	
					Computed data	55 % $C_{26}H_{54}$ 45 % $C_{27}H_{56}$	57·7°	—	49·8°	35·6	
					B. 124	**88 % $C_{26}H_{54}$** **9 % $C_{28}H_{58}$** **3 % $C_{30}H_{62}$**	**56·8–57°**	**56·6°**	**47°**	**35·51**	

The most probable constitution of each fraction is given in heavy type.

mixture, probably owing to the presence of traces of the lower paraffin in the top, and the higher in the lower fractions. We have verified this by making appropriate mixtures containing small amounts of the suggested impurity, as shown in the case of B. 114 and B. 119. There is no change in the M.P., S.P. or crystal spacing, but the transition-point is depressed about 2°. We have in each case tested the possibility of "odd + even" and "2 odd" paraffin combinations. While in some cases agreement is fair, in others it is necessary to assume only small amounts of one component, and the X-ray pictures definitely reject such a mixture. The behaviour of the material after partial fractionation, as mentioned above, suggests that it does not contain more than three components in any appreciable amount, and the three even number paraffins suggested above are the only ones that give consistent results throughout the series. Observations on the second transition-points prove that the chains do not differ by three or more carbon atoms, also if any one of the fractions is in the main composed of paraffins differing by four carbon atoms, the photographs would have shown the two sets of spacings. We consider that the above results show conclusively that the mixed primary alcohol consisted of *n*-triacontanol, *n*-octacosanol and *n*-hexacosanol.

The paraffin fraction. The crude fraction, recrystallised from carbon disulphide, melted at 63–64°. The product was slightly brown and was therefore dissolved in boiling alcohol and clarified with charcoal. The resulting product melted at 63·2–63·6°, and set at 62·4° with no definite crystal form. The material, in fact, reminded us of the crude hydrocarbon fraction of cabbage from which the ketone had not yet been separated. A portion of the product was therefore oximated and an attempt made to separate a ketoxime soluble in light petroleum, but none was found. Furthermore analysis showed that no large amount of a ketone or other oxygen-containing substance could be present in the mixture. The material was therefore repeatedly treated with sulphuric acid at 130° until no further blackening occurred. Contrary to our expectations the M.P. was not raised but was lowered to 62·6–63°. The crystal spacing was 38·7 Å. (Series No. C. 29). This spacing at the time seemed quite correct for $C_{29}H_{60}$. The M.P., moreover, agreed with that of the same material isolated by Channon and Chibnall [1929] from cabbage. But synthetic $C_{29}H_{60}$ melted at 63·4–63·6° and no amount of purification with sulphuric acid enabled us to raise the M.P. of the apple product above 63°. We were therefore forced to the conclusion that this apple product must be a mixture and in spite of this was giving a correct crystal spacing for a pure paraffin. This led us to the investigation of the crystal spacing of mixed paraffins and we then found that the spacing, for example 38·7 Å., of a pure paraffin could also be the spacing given by a mixture, as has been shown in the preceding paper [1931] dealing with these paraffins and their mixtures. We therefore attempted to fractionate the product derived from the apple and found that this could be slowly but effectively accomplished by making use of the different solubilities of the paraffins in cold petroleum (B.P. 40–60°). The fractionation, practical

details of which are given later, finally gave us a series of products whose melting-points and crystal spacings are given in Table II. The highest melting fraction, 63·4–63·6°, was further fractionated by means of petroleum (B.P. 40–60°) into three samples (C. 133–135). From the discussion which we gave in the preceding paper on the criteria necessary for judging the purity of a paraffin we consider that their melting- and transition-points and crystal spacings are definite proof that a highly pure sample of $C_{29}H_{60}$ has been isolated. When the data given in Table II are viewed as a whole it is seen that the variation in the crystal spacing with the melting-point is very similar to that which would be given by a mixture of $C_{29}H_{60}$ and $C_{27}H_{56}$. In the sixth column of Table II we have given the percentage composition calculated from the M.P. of a mixture of $C_{29}H_{60}$ and $C_{27}H_{56}$ and in column 7 the crystal spacing of such a mixture calculated from the synthetic mixture of $C_{31}H_{64}$ and $C_{29}H_{60}$ given in the previous reference. It will be observed that the suggested and observed crystal spacings are in very close agreement. We would call particular attention to fraction C. 138. Its melting-point suggests that it could be either $C_{28}H_{58}$, equimolar $C_{27}H_{56} + C_{29}H_{60}$ or 66 % $C_{29}H_{60} + 34$ % $C_{26}H_{54}$. A comparison of the crystal spacings and the transition-points shows clearly that it is equimolar $C_{27}H_{56} + C_{29}H_{60}$. Moreover we did not find, during the course of the fractionation, any product melting between 61·4–61·6° which could not be readily separated into a series of fractions having M.P. both higher and lower. We therefore draw the definite conclusion that the mixed paraffin contains only $C_{29}H_{60}$ and $C_{27}H_{56}$ and that $C_{28}H_{58}$ and $C_{26}H_{54}$ are absent.

General discussion.

Summarising the above results it will be seen that we have been able to prove definitely the presence in the apple peel wax of the following substances, *n*-nonacosane, *n*-heptacosane, *d*-10-nonacosanol, *n*-triacontanol, *n*-octacosanol and *n*-hexacosanol.

It is interesting to consider these results in relation to the hypothesis advanced by Channon and Chibnall [1929] on the metabolism of paraffins in the plant. If the paraffin (III) is synthesised from the fatty acids (I) *via* the ketone (II), then the discovery of a secondary alcohol such as *d*-10-nonacosanol together with *n*-nonacosane in the same wax is interesting. Both could, of course, be formed by reduction of the corresponding ketone, yet the reduction of —CHOH— to —CH_2— is so difficult, both chemically and by the aid of living organisms, that one cannot suggest that the alcohol is an

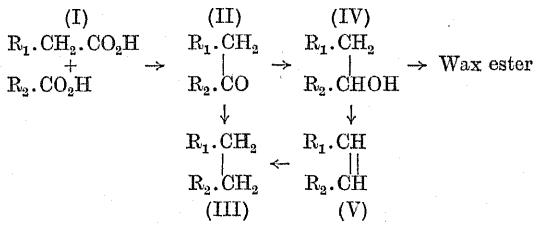


Table II. *Melting-points and crystal spacings of the paraffin fractions (with certain standards for comparison).*

Series No.	Transition-point on heating	Melting-point	Setting-point	Transition-point on cooling	Suggested composition %		Crystal spacing	
					$C_{27}H_{56}$	$C_{29}H_{60}$	Observed Å.	Calculated from Col. 6 Å.
C. 133	57·2–57·5°	63·4–63·6°	63·2°	55·8°	0	100	38·8	38·7
C. 134	57·5–57·8°	63·4–63·6°	63·2°	56·0°	0	100	38·6	38·7
C. 135	57–57·3°	63·4–63·6°	63·2°	55·3°	0	100	38·75	38·7
C. 136	55·2–55·8°	62·7–62·9°	62·3°	54·7°	17	83	38·85	38·8
C. 137	53·5–54°	61·5–61·8°	61·4°	52°	40	60	38·65	38·5
C. 138	52–52·5°	61·4–61·6°	61·2°	51·2°	44	56	38·35	38·4
C. 139	52°	60·5–60·7°	59·6°	50·4°	62	38	38·0	38·1
C. 140	51°	59·8–60·2°	58·8°	48·5°	76	24	37·4	37·4
					Composition of standards			
C. 26	57·5°	63·4–63·6°	63·2°	56·0°	$C_{29}H_{60}$		36·68	
C. 114	57·2–57·4°	61·2–61·5°	61·1°	53·7°	$C_{28}H_{58}$		37·0	
							33·25	
C. 22	52·8–53°	59·0–59·1°	56·5°	51°	$C_{27}H_{56}$		36·4	
B. 122	50–52°	60·7–61°	60·7°	Indistinct	66 % $C_{29}H_{60}$ 34 % $C_{26}H_{54}$		38·6	
B. 123	Indistinct 52–52·5°	61·1–61·3°	60·9°	51–50·5°	Equimolar $C_{27}H_{56}+C_{29}H_{60}$		38·2	

intermediary product in the direct reduction of the ketone to the paraffin. If the alcohol and paraffin have a common origin, and the fact that they both contain 29 carbon atoms is strong evidence on this point, then one would suggest (1) that the secondary alcohol had been formed by partial reduction of the ketone and that it is an alternative end-product to the hydrocarbon. It might, of course, unite with a long-chain fatty acid to form a true wax. (2) That the ketone (II) undergoes a reduction first to the secondary alcohol (IV) and then to the corresponding paraffin (III) *via* the corresponding olefine (V). Such a reduction involving the loss of water and the creation of a double bond is well known in biological reactions (*cf.* the interconversion of malic and succinic acids *via* the unsaturated fumaric acid). It is true that in the present case the ketone has not been found, but support for the hypothesis that it is the precursor of both the secondary alcohol and paraffin is obtained from a later research, in which it is shown that 15-nonacosanol, 15-nonacosanone and *n*-nonacosane are all present in the wax of the Brussels Sprout leaf. Our own researches have not yet given any definite evidence for the presence of olefines in plant waxes. There are, however, a few references in the literature to the isolation of products that appear to contain only carbon and hydrogen in the proportion close to that found in the paraffins and which have been stated to possess small iodine values. It is possible therefore that olefines corresponding with these ketones, alcohols and paraffins are present in the plant waxes.

Channon and Chibnall [1929] when they first put forward the hypothesis that the ketone might arise through the condensation of two molecules of fatty acids drew attention to the fact that the ketone they found in the cabbage leaf, 15-nonacosanone would postulate the presence in the plant at some stage in the synthesis of pentadecoic acid, and because the presence of this acid in natural products has not been clearly demonstrated, they considered that the hypothesis should be accepted with great caution. In the present instance the two fatty acids required to give the postulated intermediate ketone would be decoic and eicosanic acids, both of which have been found in plants, although neither of them can be considered a commonly occurring fatty acid. It will be seen then that the discovery of this secondary alcohol in the apple wax does, to a certain extent, support the original contention that these long-chain products can arise from the shorter fatty acids.

We still feel however that caution is necessary. We believe that for the first time some insight is being gained into the constitution of the mixed primary alcohols which we ourselves invariably find in these plant waxes. We cannot lose sight of the possibility that these primary alcohols are as closely connected with the paraffins as are the secondary products. If so, then we should expect to find some general relationships between the number of carbon atoms in the paraffins and in the primary alcohols. We have as yet very few data concerning this point, and further discussion at the present time does not seem profitable. Our chief difficulty in collecting such data lies in the fact that those waxes which contain a large proportion of prim alcohol

(*e.g.* Chinese wax, raphia wax) do not contain paraffins or secondary products, and those which contain paraffins (*e.g.* Candalilla wax) have only a small amount of primary alcohols. From the point of view of paraffin metabolism it is essential that all these be found in one and the same wax, and it is for that reason that we have thought it worth while to resolve, with much labour, the very small amount of mixed primary alcohol (5 g.) which we isolated from the apple peel.

Experimental.

Extraction of the wax. Messrs Lyons kindly provided us with a large quantity of apple peel which had been dried *in vacuo* at 40°. It was powdered in a coffee mill and then extracted with petroleum (B.P. 40–60°) in a Soxhlet for 15 hours. The extract from 8 kg. of peel was concentrated to 500 cc. and treated with two volumes of acetone. The precipitated wax was filtered off, again dissolved in the same volume of petroleum and precipitated as before. Dried *in vacuo* the crude wax (57 g.) was a yellow-brown, brittle solid of no definite M.P.

Saponification of the wax. An alcoholic solution of potassium hydroxide (170 cc. of 4·5 %) was added to the wax (57 g.) dissolved in warm benzene (300 cc.) and the mixture boiled for 3 hours on the water-bath. Water (300 cc.) was added and the mixture again heated for 10 minutes. The benzene layer was then separated, the aqueous solution extracted with benzene and the benzene solutions combined. On removal of the solvent by distillation the unsaponifiable matter was obtained as a pale brown hard wax (50 g.) M.P. 63·5°.

Treatment with phthalic anhydride. The unsaponifiable material (50 g.) was heated with phthalic anhydride (50 g.) and pyridine (20 cc.) for 18 hours at 120° in an oil-bath. In order to remove the pyridine the hot molten mixture was then poured into dilute hydrochloric acid and stirred while the mixture was heated to boiling-point. After cooling, the solid layer of phthalates was separated and heated with successive quantities of water to remove the residual phthalic anhydride. The mixture was allowed to cool and the solid cake of crude esters was collected and dissolved in warm ether (1500 cc.), giving a light brown solution. This was cautiously agitated with an excess of slightly warm aqueous solution of sodium carbonate, which caused the separation in the aqueous phase of the insoluble white sodium salts of the primary phthalic esters. The lower layer, which was an emulsion of water, ether and suspended solid matter, was separated from the supernatant ether and centrifuged. By this means the solid sodium salts collected at the ether-water interface and could be readily removed. This semi-solid mass was freed from most of the ether-soluble matter with which it was contaminated by shaking with a large volume of ether and centrifuging the resultant emulsion. The solid was then dried *in vacuo* and repeatedly extracted with dry ether. There remained a grey powder (12 g.) of no definite M.P. Many experiments on the separation of these primary phthalates have shown that the precipitated sodium salts are more granular and less gelatinous if the sodium carbonate solution is slightly warm.

Even so they are difficult to filter and can only be effectively separated by centrifuging.

After separation of these insoluble sodium salts the ether solutions were combined and the solvent removed. The residue was taken up in boiling alcohol and on cooling an impure paraffin fraction (32·2 g.) crystallised out. The mother-liquor on removal of the solvent yielded 7·2 g. of a gum which was readily soluble in cold ether, alcohol and most organic solvents, and which was the sodium salt of the secondary alcohol phthalate. The crude paraffin fraction (32·2 g.) was again heated with phthalic anhydride (15 g.)—this time without pyridine—for 18 hours at 120° and then treated as before. On shaking the ethereal solution with aqueous sodium carbonate, a slight precipitate of insoluble matter was formed. This was removed and added to the sodium salts of the primary phthalates. The ether solution was evaporated and the residue dissolved in a mixture of benzene (400 cc.) and alcohol (1600 cc.). A crystalline mass of the paraffin (23 g.) separated on cooling and on evaporation the mother-liquor yielded a further quantity (6 g.) of gummy substance which was also found to be the sodium salt of the secondary alcohol ester.

Primary alcohols. The sodium salts of the primary phthalates (12 g.) were hydrolysed by heating with a solution of potassium hydroxide (20 g.) in 95 % alcohol (100 cc.) for 18 hours. The hot solution was filtered and the crystalline mass which separated on cooling was filtered off and dried *in vacuo*. This material was then extracted many times with boiling ether, the ethereal solution filtered, and the solvent removed by distillation. The residue was taken up in petroleum (400 cc. B.P. 40–60°) and boiled with charcoal. From the clarified solution the primary alcohols separated as a white crystalline product (5·7 g.). On repeated crystallisation from various solvents, fractions, varying in M.P. from 80·7° to 82·2°, were obtained together with a small amount (0·9 g.) of crude paraffin, M.P. 62°. The discussion showing that these fractions represented mixed alcohols has already been given, p. 2099.

To obtain further evidence as to the constituent alcohols present in these mixtures they were all united and the combined material (5·0 g.) reduced *via* the iodide, to the corresponding paraffin (4·0 g.) M.P. 61–61·5°. This material was submitted to a prolonged fractionation by means of light petroleum (B.P. 40–60°) in the manner described later for the actual apple paraffin. About 250 samples were obtained during this operation, and there were finally obtained 22 samples weighing in all 1·5 g., whose melting-points and crystal spacings are given in Table I and have been fully discussed in the Introduction.

Secondary alcohols. The crude sodium salt (7·2 g.) from the first treatment with phthalic anhydride was heated with potassium hydroxide (10 g.) in alcohol (75 cc.) for 18 hours. The hot solution was filtered and the crystalline solid which separated on cooling was collected and recrystallised from petroleum (B.P. 40–60°). The product, a white crystalline solid (2·4 g.) melted at 80·7°, while the mother-liquor yielded 0·6 g. of material, M.P. 64°, which was added to the crude hydrocarbon fraction described later. The sodium salt (6 g.)

from the second phthalic anhydride treatment was saponified in the same way, and gave a white crystalline substance (2·9 g.), M.P. 80·9–81·2°, together with an impure fraction (0·65 g.) which was added to the crude paraffin fraction. The prolonged treatment with alcoholic potassium hydroxide which we used in this work to saponify the phthalates of the primary and secondary alcohols caused considerable decomposition and darkening of the solution. More recently an alternative method of hydrolysis with sodium ethoxide has been employed which gives a much better recovery of the alcohol. The ester (1 g.) dissolved in benzene (20 cc.) is heated to boiling with alcohol (10 cc.) in which sodium (0·7 g.) has been dissolved. A precipitate of sodium phthalate readily forms and after heating for 5 minutes the solution is kept for 2 hours. Water is then added and the benzene layer evaporated to dryness. Extraction of the residue by petroleum (B.P. < 40°) yields the carbinol as a white crystalline powder (0·7 g.). The two high-melting products from the soluble sodium salts mentioned above gave no depression in M.P. when mixed and appeared to be identical. To make absolutely certain that they were not still contaminated with primary alcohol they were mixed and again put through the phthalate treatment. A small precipitate of insoluble sodium primary alcohol esters was obtained. The secondary alcohol recovered by saponification (3·7 g.), M.P. 80·5–81·5°, was recrystallised from carbon disulphide, acetone and light petroleum, and was finally obtained in long white needles, M.P. 81·9–82·2°. The identification of this alcohol as *d*-10-nonacosanol has been described in some detail in the Introduction. (Found: C, 82·0; H, 14·3. $C_{29}H_{60}O$ requires C, 82·0; H, 14·2 %.) The mother-liquors after this fractionation were collected and the residue from them crystallised from a small amount of hot acetone. The material (1·25 g.), M.P. 73°, was again fractionated. It yielded 0·46 g. of alcohol, M.P. 79°, which was an impure specimen of *d*-10-nonacosanol and a series of paraffin fractions which melted between 60 and 67°. No evidence for the presence of any other secondary alcohol, for instance one with 27 carbon atoms corresponding to *n*-heptacosane, was found.

The hydrogen phthalate of the alcohol (4·85 g.) after purification was recrystallised from alcohol (charcoal) and was obtained as a white crystalline powder (5·25 g.), M.P. 54·5–54·7°. (Found: C, 77·1; H, 11·3. $C_{37}H_{65}O_4$ requires C, 77·4; H, 11·4 %.) For a 20 % solution in chloroform the observed rotation was $\alpha^{17\cdot5}_{5461} + 0{\cdot}25°$, whence $[\alpha]^{17\cdot5}_{5461} = + 0{\cdot}62°$.

d-10-Nonacosanol acetate was prepared in the usual way: M.P. 44·5–45°.

Paraffin fraction. The crude material was crystallised from benzene-alcohol (23 g.), M.P. 62·8–63·2°. (Found: C, 85·1; H, 14·7. $C_{29}H_{60}$ requires C, 85·2; H, 14·8 %.) The attempt in previous batches to obtain a pure paraffin from such a product by treatment with sulphuric acid has been described in the Introduction. To fractionate the constituent paraffins the material was stirred for 2–3 minutes at room temperature with petroleum (B.P. 40–60°) in the proportion of 10 cc. of solvent per g. of material. The solvent was filtered off, reduced in volume, and treated with warm acetone (2 : 1). The paraffin

crystallised out and was removed by filtration. This process was repeated until no more of the original material appeared to go readily into the cold solvent. In the present case there was a residue (3·7 g.) from which we were able to obtain no definite substance. Until the possible effect of saponification and treatment with phthalic anhydride on olefines has been investigated we are not examining this material further. The many fractions of paraffins obtained in this way showed a gradual rise in M.P. Each fraction was then further fractionated as follows. The material was completely dissolved by stirring at room temperature in 100 volumes of petroleum (B.P. 40–60°). The solution was then placed in an ice-chest. As it cooled the paraffin crystallised out and by using a cooled suction funnel it was possible to obtain about six successive crops of material. These were found to show a successive decrease in M.P. By dovetailing these two methods of fractionation there was obtained fairly readily a series of 10 fractions melting between 59 and 64°. These were then treated with sulphuric acid at 130° until no further charring occurred. The upper and lower fractions showed considerable charring, the middle ones practically none at all. After this treatment the products were again fractionated and there was finally obtained after some 200 fractionations in all a series of products whose melting-points and crystal spacings (Table I) have been discussed fully in the Introduction.

Fatty acids. The amount of fatty acids obtained on saponification of the original crude wax was very small. On decomposition of the sodium salts in the usual way after complete extraction of the unsaponifiable material there were obtained some 5 g. of crude fatty acid. The material was deep green and was obviously very impure, and to obtain a colourless product it had to be boiled many times with alcohol and charcoal. By fractional crystallisation there were then obtained (1) 0·65 g. of fatty acid, M.P. 82·2–82·8°, mol. wt. by titration, 470, clearly the usual mixture of high fatty acids of mean carbon content 28–30 found in many plant waxes, and (2) 2 g. of white crystalline fatty acid, M.P. 55°. These fatty acids may have been present as wax esters with long-chain alcohols or they may have been derived from the residual amount of glycerides present in the crude wax. The amount found, however, shows clearly that the major part of the alcohol present in the wax is free. In some earlier experiments the crude wax was fractionated by means of hot acetone. From a batch of 10 g. there was obtained in one case, 0·3 g. of material almost insoluble in boiling acetone which could be recrystallised from hot ethyl acetate. It melted at 79–80° and was clearly a true wax corresponding with the long-chain primary alcohol and the higher fatty acids which were afterwards isolated. (Found: C, 82·4; H, 13·8. $C_{28}H_{57}O.OC.C_{27}H_{54}$ requires C, 82·4; H, 13·7 %.)

SUMMARY.

The wax fraction of apple peel has been used to elaborate methods for separating the paraffins, ketones, primary alcohols and secondary alcohols which occur in many plant waxes.

The method of separation adopted is based on the solubility in ether and alcohol of the sodium salts of the secondary alcohol phthalates.

The following constituents of apple peel wax have been definitely identified. *n*-nonacosane, *n*-heptacosane, *d*-10-nonacosanol, *n*-hexacosanol, *n*-octacosanol and *n*-triacontanol.

The bearing of these findings on the metabolism of paraffins in the plant is discussed.

The preliminary work in this investigation was carried out at University College, London, and we wish to record our thanks to Prof. J. C. Drummond for the hospitality of his laboratory. We should also like to thank the Imperial Chemical Industries for a grant towards the expenses of this research, Dr L. H. Lampitt of Messrs Lyons for placing at our disposal large quantities of specially dried apple peel, and both Dr Hirst and Dr Anderson for examinations of optical activity.

REFERENCES.

Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Kawamura (1928). *Jap. J. Chem.* **3**, 89.
Kipping (1893). *J. Chem. Soc.* **63**, 452.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Power and Chesnut (1920). *J. Amer. Chem. Soc.* **42**, 1509.
Piper, Chibnall, Hopkins, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2072.
Rivière and Pichard (1924). *Compt. Rend. Acad. Sci.* **179**, 775.
Sando (1923). *J. Biol. Chem.* **56**, 457.

CCXXVII. THE WAX CONSTITUENTS OF FORAGE GRASSES.

I. COCKSFOOT AND PERENNIAL RYEGRASS.

By ALFRED POLLARD, ALBERT CHARLES CHIBNALL and STEPHEN HARVEY PIPER.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington, and the Wills Physical Laboratory, University of Bristol.

(*Received October 31st, 1931.*)

Introduction.

In the present paper an account is given of an investigation into the wax constituents of two very commonly occurring foliage grasses, cocksfoot (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). It has not been found possible to obtain as complete an analysis as that recorded in the previous paper dealing with apple-peel wax, but the results are of great interest because they have enabled us to investigate the constitution of a long-chain primary alcohol which is the main constituent of these two waxes.

This alcohol has been shown to be a fairly pure sample of *n*-hexacosanol. Although this substance has been assumed to be present in many natural waxes and given the name of ceryl alcohol, evidence is produced that all the samples so far recorded in the literature are mixtures which may or may not contain *n*-hexacosanol.

No paraffin was found in the cocksfoot, although a small amount was present in the ryegrass. This had M.P. 66°. The literature contains many references to paraffins melting at this temperature, and from a similarity of melting-point they were always considered to be *n*-triacontane $C_{30}H_{62}$. We shall discuss paraffins of this type more fully in a later paper dealing with many samples of paraffins isolated by previous workers, who have very kindly placed samples of their products at our disposal for further analysis. In the present case it was quite easy to show that the paraffin, M.P. 66°, was a mixture.

It is shown in the discussion later that the hippocoprosterol of Bondzynski and von Humnicki [1896] and also of Dorée and Gardner [1908] is the so-called ceryl alcohol. The latter authors also show quite conclusively by feeding experiments that hippocoprosterol passes through the intestines of herbivorous animals unchanged. One can conclude therefore that the *n*-hexacosanol found in cocksfoot and perennial ryegrass, which may be present to the extent of 1 % of the dry weight of the grass, has no feeding value.

Experimental.

Cocksfoot (Dactylis glomerata).

Preparation of leaf extract. This was prepared by a modification of the method of Chibnall and Channon [1927]. The grass (fresh weight 33 kg., dry weight 4·9 kg.) was minced with water and the green colloidal solution separated from the cell-wall material and heated to give the "coagulum," which, after pressing, was first extracted in a Soxhlet apparatus with ether and then four times successively with boiling alcohol. The residue of leaf-cell material was pressed in a Buchner press at 500 kg./cm.^{2} until no more liquid exuded. This hard press-cake was then disintegrated in a coffee-mill and extracted first with ether and then with four successive portions of boiling alcohol. All the alcoholic extracts were united, the alcohol removed by distillation *in vacuo* and the gummy residue taken up in warm ether. The cooled ethereal solution was filtered and shaken three or four times in a funnel with water. This caused the separation of much material which coloured the aqueous layer deep brown. Emulsions were also formed which were difficult to break. The final ethereal solution was then added to the two ethereal extracts mentioned above and the whole taken to dryness; weight 277 g.

Preparation of the wax. The ethereal extract was dissolved in about 1000 cc. of warm ether and treated with two volumes of acetone. The precipitated wax and phosphatide were filtered off, redissolved in a similar quantity of warm ether and again precipitated with two volumes of acetone. The resulting product was then extracted some eight times with hot acetone, each acetone extract being filtered hot. On cooling, the crude wax crystallised out and was filtered off; weight 59·6 g. (equivalent to 21·7 % of the ethereal extract and 1·2 % of the dry weight of the grass taken).

Saponification of the wax. The wax (59·6 g.) was dissolved in warm benzene (500 cc.), a 10 % alcoholic solution of potassium hydroxide (300 cc.) added and the mixture boiled for 2½ hours on a water-bath. Warm water (700 cc.) was then added, the benzene solution separated in a funnel and the aqueous layer extracted several times with benzene. Emulsions formed during the extraction were readily broken by centrifuging. The aqueous alkaline solution after acidification gave only a trace of fatty acids. The benzene solution on evaporation gave a dark green viscous mass. To separate the unsaponifiable material from the potassium soaps of higher fatty acids the whole was extracted many times with boiling acetone, each extract being filtered hot. On cooling the unsaponifiable portion crystallised out and was removed by filtration. It was a hard greenish-yellow wax (45 g.).

Fatty acids. After extraction with hot acetone there remained a dark green residue. This material was heated with a small quantity of concentrated HCl, washed and again extracted with hot acetone. The hot filtered extracts deposited on cooling a dark green powder (7·75 g.) which appeared to be a mixture of higher fatty acids and the degradation products of chlorophyll. The

mother-liquors gave a residue of crude fatty acids (4·5 g.) which on extraction with petroleum (B.P. < 40°) gave a pale brown semi-solid mass of fatty acid (3·6 g.) similar to that given by saponification of the glyceride fraction. The dark green powder mentioned above was extracted in a Soxhlet apparatus with petroleum (B.P. < 40°). The extract yielded a pale brown substance (3·1 g.), M.P. 73–73·5°. This was saponified by heating with sodium ethoxide (0·5 g. sodium in 20 cc. of alcohol) and benzene (30 cc.) for $2\frac{1}{2}$ hours. The benzene solution was separated, the solvent removed and the residue taken up in acetone. The acetone gave on evaporation a brown residue 0·15 g. The substance undissolved by the acetone was acidified with hydrochloric acid. It was now completely soluble in hot acetone. On cooling a pale yellow granular crystalline material separated (1·9 g.), M.P. 78·2–78·7°. Further purification yielded a white crystalline granular powder, M.P. 79·4–79·8°, mol. wt. by titration 440; $C_{29}H_{58}O_2$ requires 435. It was clearly the usual "cerotic acid" of plant extracts, which was shown to be a mixture by Francis, Piper and Malkin [1930].

Treatment of the unsaponifiable material with phthalic anhydride. The material (45 g.) was heated with phthalic anhydride (45 g.) and pyridine (20 cc.) at 120° for 18 hours and, after removal of pyridine and excess phthalic anhydride in the usual way, the product was dissolved in ether and shaken with a warm solution of sodium carbonate. A copious precipitate of the sodium salts of the primary phthalates was obtained. This was washed in the usual way by shaking with ether and centrifuging, dried in the air and then extracted many times by shaking with ether. The original ethereal extract and the washings were collected, the solvent was removed and the product extracted with boiling alcohol. A small residue (1·7 g.) remained undissolved. The alcoholic solution on cooling deposited a pale green amorphous solid (7·9 g.). The alcohol mother-liquor—which should contain the soluble sodium salts of any secondary alcohol esters—was taken to dryness, but the semi-solid residue (1·2 g.) did not yield any alcohol on saponification. It was presumably a residual amount of that fraction of the unsaponifiable material, the main bulk of which, in the preparation of the crude wax, was soluble in the ether-acetone mixture. The amorphous green powder mentioned above was extracted at room temperature with cold petroleum (B.P. < 40°). The extracts were concentrated and treated with two volumes of acetone; the material which separated (3·15 g.) was removed by filtration, M.P. 59–59·5°. (Found: C, 81·6; H, 13·5 %. Iodine value, 19.) This material, which may contain unsaturated hydrocarbons, is still under investigation.

Primary alcohols. The insoluble white sodium salts of the primary phthalates (45·8 g.) were dissolved in boiling benzene (500 cc.). Sodium ethoxide (25 g. sodium and 350 cc. alcohol) was added and the boiling continued for 5 minutes. The mixture was allowed to cool and to stand for 2 hours. Warm water (500 cc.) was then added and the benzene solution separated in a funnel. The aqueous extract was extracted repeatedly with benzene. The benzene extracts

were combined, the solvent removed by distillation and the brown residue extracted with acetone and ether. There remained an insoluble residue (2·2 g.). The acetone and ethereal extracts gave 27·5 g. of brown crystalline powder (80 % of theory for $C_{26}H_{53}O.CO.C_6H_4CO_2Na$). The crude primary alcohol was redissolved in boiling acetone, clarified with charcoal, and the product which separated on cooling recrystallised first from carbon disulphide and then from acetone; yield 20·7 g. of fine white needles, M.P. 79·3–79·6°. (Found: C, 81·5; H, 14·3. $C_{26}H_{54}O$ requires C, 81·6; H, 14·2 %.) The M.P. of this primary alcohol suggested that it was the usual "ceryl" alcohol which often occurs as a constituent of plant waxes. A sample was oxidised by means of chromium trioxide in acetic acid to the corresponding acid. This had M.P. 86·5–86·8°, some degrees higher than that usually found for the cerotic acid obtained on oxidation of ceryl alcohol. Another sample was reduced *via* the iodide to the corresponding paraffin which melted at 56·3–56·6°. Synthetic *n*-hexacosane has M.P. 56·4–56·6° and, in the early stages of the research, this seemed to us evidence that we were dealing with a fairly pure sample of a primary alcohol containing 26 carbon atoms. At a later period, when our knowledge of the synthetic and mixed paraffins was more complete, it became evident that the paraffin derived from this alcohol may not be pure *n*-hexacosane but a mixture. 2·9 g. of this paraffin were therefore fractionated by means of cold petroleum (B.P. 40–60°) in the way previously described for the resolution of the constituent paraffins of apple wax. Five fractions were finally obtained whose M.P. and crystal spacings are given in Table I. It will be seen that the original paraffin was indeed a mixture but that it consisted in very large part of *n*-hexacosane because,

Table I. *Fractionation of the paraffin obtained from the crude primary alcohol.*

Series number	M.P.	Crystal spacing Å.
205	56·2–56·4°	35·5
206	56·4–56·6°	34·8 32·75
207	56·4–56·6°	35·25 32·2
208	56·4–56·6°	35·1
209	56·5–56·8°	35·5

in two of the fractions, Nos. 206 and 207, the lower spacing for this paraffin appears and the extreme limits of melting-point given by Nos. 205 and 209 do not suggest that much paraffin of higher or lower carbon content was present. Leaving for later discussion the question of whether ceryl alcohol is $C_{26}H_{54}O$ or $C_{27}H_{56}O$ the data given above showed that we were dealing with a far purer sample of *n*-hexacosanol than had hitherto been isolated from natural products. An attempt was therefore made to fractionate this grass alcohol and obtain a specimen of *n*-hexacosanol comparable in melting-point and crystal spacing with the synthetic product.

Fractionation of the primary alcohol. The alcohol (18 g.) was converted into the acetate by boiling overnight with acetic anhydride (250 cc.) and fused

sodium acetate (20 g.). The product was then poured into hot water, stirred mechanically for an hour, collected, taken up in methyl alcohol, boiled with charcoal, and the product finally crystallised from benzene; yield 17·8 g., M.P. 59·7–60°. This acetate was distilled in an apparatus designed specially for the distillation in a high vacuum of substances of high setting-point, which will be described in a separate communication. A Willstätter flask of 100 cc. capacity was employed in the first distillation. At the stage when 6·9 g. remained in the flask the distillation was stopped and the flask replaced by one of 50 cc. capacity. This residual 6·9 g. was then distilled. Throughout both distillations the temperature of the metal-bath was some 70° above that of the vapour passing over. The fractionations obtained are set out fully in Table II in which data concerning the resultant alcohols, and the paraffins, acids and esters derived from them, are also given.

Table II. *Fractionation of the crude primary alcohol.*

No.	B.P./mm.	Wt. g.	M.P.	Alcohol M.P.	Acid M.P.	Ester M.P.	Paraffin M.P.
1	186–189°/0·02	0·51	59·2–59·4°	79·4–79·8°	87·2–87·4°	—	56·2–56·4°
2	190–192°/0·02	1·02	59·4–59·6°	79·5–80°	87·2–87·5°	—	—
3	191·5–192·5°/0·02	2·44	59·4–59·5°	—	—	—	—
4	192–193°/0·017	2·05	59·5–59·6°	79·5–79·8°	87·7–87·9°	59·7–59·8°	56·4–56·6°
5	192°/0·017	1·02	59·4–59·6°	—	—	—	—
6	192–190°/0·017	3·65	59·4–59·6°	—	—	—	—
7	Residue redistilled	6·9	59·8–60°	—	—	—	—
8	196°/0·01	0·66	59·4–59·6°	79·5–79·7°	—	—	—
9	196–193°/0·005	1·04	59·5–59·7°	—	—	—	—
10	193–194°/0·006	1·55	59·3–59·5°	—	—	—	—
11	194–194·5°/0·006	1·5	59·5–59·7°	79–79·5°	86·6–87°	—	—
12	195°/0·005	0·5	59·3–59·5°	—	—	—	—
13	Residue	1·3	59·4–59·7°	78·6–78·8°	84·7–85·3°	—	56·7–56·9°

Each fraction was hydrolysed to the resulting alcohol by saponification with 10 % alcoholic potash for 2 hours, and the white crystalline solid which separated on cooling crystallised from petroleum (B.P. 40–60°). In the case of fractions 3 to 11 inclusive the alcohol, when crystallised from this solvent, came out in large thin leaflets up to 1 cm. in length. Certain of these alcohols (fraction No. 4 will be taken as an example) were then converted into the acids, esters and paraffins in the following way.

The alcohol (0·9 g.) was dissolved in warm glacial acetic acid (40 cc.) and the solution of chromium trioxide (1 g.) in glacial acetic acid (10 cc.) added in small amounts during the course of a quarter of an hour. After standing for a further half-hour the solution was poured into water, which was then extracted with warm benzene. The solvent was removed, the residue taken up in ether and filtered. The ethereal solution was shaken with a solution of sodium ethoxide and the granular sodium salt of the fatty acid separated by centrifuging. This was washed with ether, decomposed with HCl, and the free acid extracted with ether. The resulting product was crystallised four times from acetone, M.P. 86·3–86·8°. (Found: C, 78·4; H, 13·3. $C_{26}H_{52}O_2$ requires C, 78·7; H, 13·2 %.) Recrystallised from pyridine and then four times successively from acetone,

the acid melted at 87·7–87·9°. The ethyl ester, recrystallised repeatedly from acetone, was obtained in long flat needles about 1 cm. in length, M.P. 59·7–59·8°. (Found: C, 79·2; H, 13·5. $C_{28}H_{56}O_2$ requires C, 79·3; H, 13·3 %.)

Another portion of the alcohol was converted into the iodide. This was reduced to the corresponding paraffin by a method used by Levene *et al.* [1915] in place of that previously employed by us, *viz.* amalgamated zinc wool and alcoholic HCl. The iodide (0·9 g.) was dissolved in glacial acetic acid (10 cc.), zinc dust (1 g.) added, and the mixture boiled over a small free flame. Every two hours a stream of dry HCl was passed in for about half a minute. At the end of two working days the zinc had dissolved. The mixture was diluted with water and a layer of paraffin (0·6 g.) collected. This was purified by treatment with sulphuric acid at 130° and crystallised from benzene-alcohol (1 : 2), M.P. 56·4–56·6°. This paraffin was then fractionated by means of cold petroleum (B.P. 40–60°) into six fractions whose M.P. and crystal spacings are given in Table III. The most striking feature of the results is the appearance of the

Table III. *Fractionation of the paraffin obtained from the purified primary alcohol.*

Series number	Transition-point on melting	M.P.	S.P.	Transition-point on cooling	Crystal spacing Å. *A*	*B*	*C*
B 72	50·5–51·5°	56·4–56·6°	56·2°	48·1°	34·6	—	30·9
B 73	51–52°	56·4–56·6°	56·1°	48·2°	—	32·65	30·9
B 74	51–52°	56·4–56·6°	56·1°	48·2°	—	—	30·9
B 75	51–52°	56·4–56·6°	56·1°	48·2°	—	—	30·9
B 76	51–52°	56·4–56·6°	56·1°	48·2°	—	—	31·0
B 77	51–52°	56·4–56·6°	56·1°	48·1°	—	32·6	31·0
$C_{26}H_{54}$	51·5–52°	56·4–56·6°	56·1°	48·3°	34·95	32·6*	31·0

spacing in the column headed *B*. Although such a spacing has not been observed with synthetic hexacosane the value 32·6 falls exactly at the right place on the extrapolated *B* line. The extrapolated value for the *A* (vertical chain spacing) of $C_{24}H_{50}$ is 32·5. Now $C_{26}H_{54}$ is the first of the long paraffins to fail to appear in the *B* modification. It is therefore likely that the presence of a small impurity, having a normal spacing with the value which hexacosane would take in the *B* modification, will be able to force this crystal form upon it. We conclude that there is a small contamination of $C_{24}H_{50}$. The depression of the transition-point on cooling for five of the middle fractions is only 0·1°. As a comparison we know that 1 % of hexacosane in octacosane depresses the transition-point on cooling by 0·6° and on heating by 0·5°. We are therefore confident that there is much less than 1 % of tetracosane in these mixtures.

If this is so then the alcohol of Fraction 4 is *n*-hexacosanol with less than 1 % *n*-tetracosanol. Unfortunately at the present time this alcohol has not been synthesised, so that direct confirmatory data are lacking. We can, however, obtain further evidence for the purity of the grass product by considering the M.P. and crystal spacings of the alcohol itself and the derived acid and its ester. The alcohol melted at 79·8° and the crystal spacing (Series B, No. 33) was

* "Extrapolated value."

57·8 Å. From the data of Malkin [1930] extrapolated values for *n*-hexacosanol would be M.P. about 80° and crystal spacing 57·9 Å. The acid melted at 87·8°. The M.P. of synthetic *n*-hexacosanic acid was found by Francis, Piper and Malkin [1930] to be 88·2°, but they state quite clearly that this point is 0·5° higher than they would expect from their curve showing the M.P. of the even number acids. The crystal spacings for the synthetic acid were 63·3 Å. for the *B* and 57·3 Å. for the *C* spacings. The spacings obtained for the acid derived from the grass alcohol were (Series B, No. 35) 62·5 Å. and 56·7 Å. respectively. The synthetic ethyl ester melted at 59·6° and had a crystal spacing of 34·64 Å. The ethyl ester derived from the grass alcohol melted at 59·8° and had a crystal spacing (Series B, No. 37) of 34·4 Å.

There are no data for mixed even number acids of more than 18 carbon atoms, but it is to be expected that a shorter chain would lower both *B* and *C* spacings of hexacosanic acid. The results of the measurements on the acid from the grass alcohol are to this extent in accord with the deductions made for the derived paraffin—namely, that a small amount of tetracosanic acid is present. The hexacosanic acid was not available in sufficient amount to purify *via* the ester, and cannot therefore be fairly compared with that of Francis, which had been through this process. Similar conclusions may be drawn from the spacings of the ester.

The alcohol of Fraction 1, M.P. 79·4–79·6°, gave an acid, M.P. 87·2–87·4°, and a paraffin, M.P. 56·2–56·3°, which was fractionated with light petroleum. The results are given in Table IV. The high M.P. of the acid and the narrow

Table IV. *Fractionation of the paraffin obtained from the highest and lowest boiling fractions of the crude primary alcohol acetates* (cf. *Table II*).

Fraction number	Series number	Transition-point	M.P.	S.P.	Crystal spacings Å. *A*	*B*	*C*
1	B 101	51–52°	56·3–56·5°	56·0°	—	32·6	—
	B 102	49–50°	56·2–56·4°	55·8°	—	32·6	—
	B 103	48·5–51·5°	56·2–56·3°	55·8°	30·8	—	34·6
	B 104	48–50°	56·2–56·4°	55·8°	30·8	—	34·6
13	B 105	43–44°	56·8–57°	56·8°	—	—	—
	B 106	43–45°	56·8–57·1°	56·6°	—	—	—
	B 107	43–45°	56·7–57°	56·5°	—	—	—
	B 108	44·5–45·5°	56·4–56·7°	56·4°	—	—	—

range within which the paraffins melt show that this lower fraction consists in very large part of *n*-hexacosanol. The *B* spacing again suggests the presence of a small amount of $C_{24}H_{50}$, about 1 % in B 103 and 104, much less in B 101 and 102. It is interesting to note that the depression in the transition-temperature of about 2° with a change in M.P. of only 0·1–0·2° would have suggested to us, from a consideration of the data given in the preceding paper, that the impurity was not *n*-pentacosane but tetracosane (compare series for C_{28}–C_{29} and C_{26}–C_{28}). The alcohol from the residue (Fraction 13) obviously contained alcohols higher than *n*-hexacosanol. The low transition-temperatures recorded

in Table IV for the derived paraffins show that these contain a small amount of a mixture of at least two other higher paraffins, in the case of B 105 as much as 10 %.

Perennial ryegrass (Lolium perenne).

Preparation and saponification of the wax. The wax was prepared and treated in a similar way to that described for the products from cocksfoot. In the case of the ryegrass the wax amounted to only 10 % of the total ethereal extract, or 0·38 % of the dry weight of the grass.

14·3 g. of the wax were saponified as before and yielded 2·6 g. of fatty acids, M.P. 80–81°, together with an unsaponifiable fraction which, after crystallisation from acetone and decolorising with charcoal, weighed 8·6 g. This material yielded an insoluble sodium salt of a primary phthalate (6·8 g.) and a crude paraffin fraction (2·1 g.).

Primary alcohol. The insoluble sodium salt was hydrolysed with sodium ethoxide in benzene-alcohol and yielded 4·2 g. of white crystalline primary alcohol, M.P. 78°. Fractional crystallisation from light petroleum, carbon disulphide and acetone gave two products.

(1) (2·4 g.) M.P. 79·8–80°; crystal spacing (Series C, No. 57), 58·4 Å. It gave on oxidation an acid, M.P. 85–85·5°, and on reduction *via* the iodide a paraffin, M.P. 56·5–56·7°; crystal spacing (Series C, No. 58), 35·5 Å.

(2) (1·2 g.) M.P. 79–79·3°; crystal spacing (Series C, No. 39), 58·2 Å.; acetate, M.P. 60·2–60·5°; acid, M.P. 84·5–84·8°; and paraffin, M.P. 56·4–56·6°. The crystal spacings of this paraffin (Series C, No. 24) were 35·5 and 32·5 Å.

The M.P. and crystal spacings of the derived paraffins suggest that these alcohol fractions consist in large part of *n*-hexacosanol, but the amount of longer-chain alcohols present is greater than in the case of the corresponding product from cocksfoot.

Paraffin fraction. No trace of a secondary alcohol or a ketone could be found. The crude paraffin was decolorised with charcoal in 95 % alcohol and then treated with sulphuric acid; M.P. 65·9–66°, transition-temperature indistinct. Fractional crystallisation from petroleum gave without any trouble the samples recorded in Table V. It will be seen that the paraffin is a complex

Table V. *Fractionation of the paraffin, M.P. 66°, isolated from ryegrass.*

Series number	Heating transition-point	M.P.	S.P.	Cooling transition-point	Crystal spacing Å.
B 22	50·5–51·5°	62·3–62·6°	62·0°	50–49·5°	39·7
B 23	55–56°	65·0–65·5°	64·5°	54–53°	40·75
B 24	57–58°	66·7–66·8°	66·5°	57–56°	42·3
B 25	60–61°	67·9–68·1°	67·8°	60–59°	43·0
B 26	61–62°	68·4–68·8°	68·4°	61–60°	43·5

mixture and its composition cannot even be suggested. The amount isolated was small, and as it is impossible to obtain large samples of a given species of grass which can be considered free from all other grasses we do not consider

the present wax a suitable raw material for experiments on the separation of mixed paraffins in plant waxes. The fact that the properties of this crude paraffin agree with those ascribed by many writers to *n*-triacontane has been briefly mentioned in the Introduction.

Discussion.

It will be seen that the fractionation of these grass waxes has yielded in large part a fairly pure specimen of a long-chain primary alcohol. There were also obtained certain products whose nature has not yet been definitely determined and only a small amount of higher fatty acids. The amount of true wax, therefore, must have been relatively small, and the major part of the primary alcohol must have been uncombined. The fact that this primary alcohol was practically a pure specimen of *n*-hexacosanol allows us to discuss in some detail the constitution of the alcohol termed "ceryl," which was originally isolated from Chinese wax, and the hippocoprosterol of Bondzynski and von Humnicki [1896].

Ceryl alcohol. The data recorded by previous workers are set out fairly fully in Table VI. There are numerous other references to the isolation of this

Table VI. *Collected data concerning ceryl alcohol.*

Reference	Source	Alcohol M.P.	Acetate M.P.	Acid M.P.	Ester M.P.	Paraffin M.P.
This paper	Cocksfoot crude alcohol	79·6°	59·7–60°	86·8°	—	56·3–56·6°
This paper	Cocksfoot purified alcohol*	79·7°	59·5–59·6°	87·7–87·9°	59·7–59·9°	56·4–56·6°
This paper	Perennial ryegrass	79·3°	60·2°	84·5–84·8°	—	56·5–56·7°
Francis *et al.* [1930]	Chinese wax	79°	60°	82°	60°	—
,,	,,	83°	65–66°	83·8°	64·2°	—
Levene *et al.* [1915]	,,	80°	64–65°	—	—	61°
Gascard [1921]	,,	79·5–80°	—	82–82·5°	—	59·5°
This paper	,,	78·7°	62°	83–83·2°	—	59–59·5°
Brodie [1848]	Beeswax	79°	—	81°	—	—
Schwalb [1886]	,,	79°	—	78·5°	—	—
Stürcke [1884]	Carnauba wax	76°	—	—	—	—
Benedikt and Ulzer [1886]	Seed lac	79·5°	65°	—	—	—
Collison and MacLean [1931]	Spinach leaves	77–78°	61·5°	—	—	—
Stern and Zellner [1926]	Bark of *Sonchus arvensis*	80°	63·4°	—	—	—
Bareuther [1923]	Sunflower oil	81·2°	63·5°	77·6°	—	—
Chibnall *et al.* [1931]	Apple-peel wax	80·2–80·7°	64·5–64·8°	83–83·5°	—	60–60·2°

* Shown to be *n*-hexacosanol with less than 1 % impurity.

alcohol from plant sources, but in these cases the M.P. of derivatives have not been quoted. The M.P. of the alcohol was generally recorded as 79–80°, of the acetate as 64–65°, and of the acid obtained on oxidation as 81–82°. Titration of the acid suggested to earlier workers that it contained 26 carbon atoms. The low M.P., however, was a puzzle because the extrapolated melting-point of *n*-$C_{26}H_{52}O_2$ was many degrees higher than that actually found. Many workers, therefore, suggested that this and similar alcohols, such as myricyl alcohol, contained a branched chain, and that this was the reason for the low M.P. of the acid. Levene *et al.* [1915] obtained a specimen of ceryl alcohol, M.P. 80°, giving an acetate, M.P. 64–65°, and by reduction of the corresponding iodide a paraffin,

M.P. 61°. This latter was higher than that given by synthetic *n*-hexacosane and they concluded that the ceryl alcohol must have a branched and not a normal carbon chain. A more complete investigation of ceryl alcohol from Chinese wax was made by Gascard [1921]. By fractionation he obtained what he thought was pure ceryl alcohol, M.P. 79·5–80°, which yielded on oxidation a specimen of cerotic acid, M.P. 82–82·5°. The molecular weight of this acid by titration suggested that it contained 27 carbon atoms. To confirm this finding he reduced the alcohol *via* the iodide to the corresponding paraffin. This melted at 59·5°, the same temperature as the M.P. of *n*-heptacosane previously synthesised by Kraft. Gascard, therefore, concluded that ceryl alcohol was $C_{27}H_{56}O$.

More recently, Francis, Piper and Malkin [1930] fractionated, by distillation of the acetate, a specimen of ceryl alcohol from Chinese wax and separated two samples, one melting at 79° and the other at 83°. The corresponding acids melted at 82° and 83·8° respectively. An examination of the crystal spacings of the acids suggested that the former was a mixture of C_{26} and C_{28}, while the latter was C_{28} with probably an even higher acid. No evidence of branched chain acids was obtained, and they concluded that ceryl alcohol from Chinese wax was a mixture of primary alcohols. With this finding we are in complete agreement, and our own researches enabled us to reconcile some of the earlier conflicting results.

In the first place a specimen of ceryl alcohol, M.P. 79·7°, obtained from Chinese wax, gave an acetate, M.P. 62°, an acid, M.P. 83–83·2°, and a paraffin, M.P. 59–59·5°. These values it will be observed are in agreement with those of Gascard. The crystal spacing (Series C, No. 44) of the paraffin, however, gave only three very diffuse orders measuring 36·7 Å. It was clearly a mixture of more than two paraffins, showing that the ceryl alcohol was itself a complex mixture of mean molecular weight corresponding to C_{27}.

We have in the present research isolated what the earlier workers obviously considered to be ceryl alcohol. Table V shows that the M.P. of the acid derived from both the crude and purified grass alcohol is some 4° higher than that recorded by any previous worker. The acetate, on the contrary, melts some 2–4° lower. As the grass alcohol has been shown to be nearly pure *n*-hexacosanol it is clear that all the other alcohols quoted in Table V were mixtures of alcohols which, on oxidation, have given a mixture of acids showing a depressed M.P. Data as to the melting-points of the pure and mixed acetates of the higher primary alcohols are lacking, but it is clear that those of mixed acetates exhibit very much less depression than those of the corresponding acids.

The actual isolation of *n*-hexacosanol from natural sources and the definite proof that the ceryl alcohol of former workers was neither *n*-hexacosanol nor *n*-heptacosanol but a mixture of primary alcohols of the *n*-fatty acids necessitate a new definition of the name ceryl alcohol. Applied originally to the alcohol, M.P. 80°, isolated from Chinese wax, it has unfortunately always been applied to products now shown to be mixtures. Such mixtures will no doubt be encountered in future researches and a collective name will still be required

for them. We suggest that the name ceryl alcohol be retained for this purpose and that, if further fractionation should lead to the isolation of pure primary alcohols of the *n*-fatty acids, these should be referred to by their chemical names. On this new definition the alcohol isolated in the present research from cocksfoot is *n*-hexacosanol and not ceryl alcohol.

Hippocoprosterol. This substance, M.P. 74·5°, was discovered by Bondzynski and von Humnicki [1896] in the faeces of the horse and was supposed to have been formed by reduction of cholesterol in the intestine. The product was further examined by Wilenko [1906] who, by fractional crystallisation from 97 % alcohol, obtained two isomeric compounds, M.P. 67° and 56°, which he designated as α- and β-hippocholesterol respectively. Dorée and Gardner [1908] were unable to substantiate Wilenko's findings and showed that the hippocoprosterol was a saturated alcohol, M.P. 79°, which gave an acetate, M.P. 61–61·2°, and no sterol reactions. The same material was found in the excrement of the cow and sheep and also in the ether extract of grass itself. They therefore drew the conclusion that hippocoprosterol was not a product of animal metabolism but was a constituent of the grass taken as food which had passed unchanged through the animal.

From the results given in the present paper there is no doubt that hippocoprosterol, M.P. 79°, acetate, M.P. 61·2°, is ceryl alcohol. Ordinary forage grass is a mixed herbage, and there is no reason to suppose that all the component grasses contain nearly pure *n*-hexacosanol as do cocksfoot and perennial ryegrass. Furthermore some of these grasses may contain a larger quantity of a paraffin than ryegrass. A mixture of paraffins and ceryl alcohol is not readily separated by crystallisation from alcohol, and in that fact seems to us to lie the explanation of Wilenko's low M.P. We attribute the differences between Dorée and Gardner's and Wilenko's results therefore to a difference in the herbage mixture.

Summary.

The wax fractions of two common forage grasses, cocksfoot and perennial ryegrass, have been investigated.

In the case of cocksfoot the chief constituent is a long-chain primary alcohol which has been shown to be nearly pure *n*-hexacosanol. It contains less than 1 % of *n*-tetracosanol, and about an equal amount of an unidentified longer-chain alcohol.

The same alcohol is present in ryegrass wax, but the amount of impurities is greater.

The constitution of the so-called ceryl alcohol is discussed, and the recommendation made that the term be restricted in future to the mixed primary alcohols, M.P. *ca.* 80°, which often occur in plant waxes.

The probable identity of hippocoprosterol with ceryl alcohol is discussed.

From previous work on hippocoprosterol it is concluded that the wax fractions of both these forage grasses have no feeding value.

We should like to record our thanks to the Imperial Chemical Industries for a grant to cover the cost of this research, and their staff at Jealott's Hill Research Station, Bracknell, for the supply of pure strain grasses.

REFERENCES.

Bareuther (1923). *Chem. Umschau.* **30**, 117.
Benedikt and Ulzer (1866). *Monatsh.* **9**, 581.
Bondzynski and von Humnicki (1896). *Z. physiol. Chem.* **22**, 396.
Brodie (1848). *Liebig's Ann.* **67**, 180.
Chibnall and Channon (1927). *Biochem. J.* **21**, 225.
—— Piper, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2095.
Collison and MacLean (1931). *Biochem. J.* **25**, 2, 606.
Dorée and Gardner (1908). *Proc. Roy. Soc. Lond.* B **80**, 212.
Francis, Piper and Malkin (1930). *Proc. Roy. Soc. Lond.* A **128**, 214.
Gascard (1921). *Ann. Chim.* (9), **15**, 332.
Levene, West and Van der Scheer (1915). *J. Biol. Chem.* **20**, 521.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Schwalb (1886). *Liebig's Ann.* **235**, 106.
Stern and Zellner (1926). *Monatsh.* **46**, 461.
Stürcke (1884). *Liebig's Ann.* **223**, 283.
Wilenko (1906). *Bull. Internat. Acad. Sci. Cracovie*, **1**, 20.

CCXXVIII. THE DICARBOXYLIC ACID-NITROGEN OF PROTEINS.

WITH A NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

BY MANAYATH DAMODARAN.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received October 31st, 1931.*)

THE usual procedure for the Van Slyke determination of the nitrogen distribution of proteins [Plimmer, 1917] makes no provision for the dicarboxylic acids. A modification was described by Andersen and Roed-Muller [1915] in which the dicarboxylic acids were determined indirectly by the base bound at neutrality to phenolphthalein. With the only two proteins investigated by these authors the method gave satisfactory results but, involving as it does the complete absence of calcium salts and the previous removal of the diamino-acids, it calls for considerable modification of the ordinary Van Slyke procedure by which all the known proteins have been analysed. The presence of ammonia, of glucosamine or of organic acids was also shown to give rise to error. Probably for these reasons the method of Andersen and Roed-Muller has found no general application.

In the course of many protein analyses carried out in this laboratory it has been found that the lime-alcohol method of Foreman [1914] for precipitating the dicarboxylic acids gave very satisfactory results and that it could be easily incorporated into the ordinary procedure for Van Slyke analyses, so that the total dicarboxylic acids could be rapidly determined when working with only small quantities of material and without actual isolation of the amino-acids themselves.

The addition of this new group to the usual nitrogen distribution scheme is considered all the more desirable in view of the many errors to which the determination of the bases has been shown to be liable. The separation of the bases by means of phosphotungstic acid is neither quantitative nor specific [Thimann, 1930; Plimmer and Rosedale, 1925, 1; Gortner and Sandstrøm, 1925]. According to Plimmer [1916], the decomposition of histidine, amounting to 3–4 %, tends to give a higher value for arginine. To this must be added the possible presence in protein hydrolysates of the newly discovered amino-acid, citrulline [Wada, 1930], which also decomposes, giving ammonia on treatment with alkali. Finally it has to be remembered that peptides and, under certain

conditions, amino-acids such as phenylalanine and proline, are precipitated by phosphotungstic acid.

Apart from widening the scope of the Van Slyke method of analysis and providing an additional criterion for the characterisation of proteins in general, a knowledge of the dicarboxylic acid content of plant proteins is of importance on account of the possibly predominant rôle played by asparagine and glutamine in plant metabolism [Onslow, 1930].

The technique previously adopted in this laboratory was the modification of the method of Foreman [1914] described by Kingston and Schryver [1924] wherein barium hydroxide was substituted for calcium hydroxide. This modification is convenient in dealing with large amounts of protein as the quantity of alcohol required is much smaller, but it has the disadvantage that the amount of extraneous matter precipitated is appreciable [Jones and Moeller, 1928; Damodaran, 1931]. It was therefore decided to attempt to apply Foreman's original procedure to the Van Slyke method of small scale analyses.

Experiments were carried out on the following lines. (i) The completeness of the precipitation by alcohol of the calcium salts of glutamic and aspartic acids when these were present in small amount was tested. (ii) As the dicarboxylic acid precipitate is known to be contaminated with a small amount of material precipitable by phosphotungstic acid it was necessary to see if due allowance could easily be made for such material and a reasonably correct figure thus obtained for the basic N. (iii) When protein hydrolysates are treated by the Foreman method it is found that, after apparently complete precipitation of the dicarboxylic acids (as tested by adding small amounts of alcohol), a further flocculent precipitate always forms slowly either on standing for a long time or on the addition of large volumes of alcohol. With caseinogen Foreman showed that this precipitate contained no dicarboxylic acids. It was considered desirable to verify this with at least one other protein of a different class and to this end experiments were carried out on gliadin. (iv) Experiments were made on gliadin to see if reprecipitation were necessary when using calcium hydroxide. (v) The new modification was finally tested on five proteins whose dicarboxylic acid contents have been definitely determined by large scale analyses and was then applied to a number of proteins of different classes, the dicarboxylic acid contents of which are either unknown or not accurately known.

Experimental.

Completeness of the precipitation of glutamic and aspartic acids by calcium hydroxide and alcohol. 5 cc. of a solution of glutamic acid hydrochloride containing 0·1 g. of glutamic acid were made alkaline with calcium hydroxide and treated with 95 % alcohol till precipitation was complete, approximately 50 cc. being used. The precipitate was filtered at the pump and washed three times with 20 cc. portions of 95 % alcohol; 98·8 % of the glutamic acid was precipitated. A similar experiment with 5 cc. of a 0·2 % solution of aspartic acid showed that 99·1 % of the acid was precipitated.

Analysis of gliadin. It will be convenient here to describe in full the modified Foreman method as applied to gliadin.

Hydrolysis. 6 g. of gliadin (for duplicate analyses) were hydrolysed by boiling with 20 % hydrochloric acid for 18 hours. On an aliquot total N and amide-N were determined.

Amide-N. It was found convenient to determine the amide-N by distilling in steam a small aliquot of the hydrolysate with sodium hydroxide in a Parnas-Wagner micro-distillation apparatus for 4½ minutes. In most cases slightly higher results were obtained than those previously recorded, but this can hardly be ascribed to decomposition of arginine or other amino-acids as the period of distillation involved is so short.

The main fraction of the hydrolysate was next divided into two portions each of which was treated in the following way.

Removal of humin and ammonia. The solution was made strongly alkaline with calcium hydroxide (10 % suspension), the precipitated humin with the excess of calcium oxide centrifuged off and washed free from chlorides (three washings each with 50 cc. of warm water were usually required). The solution with the washings was evaporated *in vacuo* at 40° to a volume of 10–15 cc. The air admitted during distillation was freed from carbon dioxide by means of a soda-lime tube.

Precipitation of the dicarboxylic acids. To the syrup in the flask about 10 vols. of 95 % alcohol were added very slowly in small quantities at a time, with vigorous stirring, till precipitation was complete. This point was determined by transferring the mixture to a 250 cc. centrifuge-tube, centrifuging and testing by the addition of 10 cc. portions of alcohol. When no further precipitate formed the clear liquid was poured off and the precipitate, some of which adhered to the sides of the flask, was washed twice with 50 cc. portions of 95 % alcohol.

Treatment of the dicarboxylic precipitate. The precipitate was dissolved in a little acidulated water and the solution after making up to 25 cc. was analysed for nitrogen. The value obtained was 28·6 % of the total N. The remainder of the solution was transferred to a centrifuge-tube, and treated with 5 cc. of concentrated hydrochloric acid and a saturated aqueous solution of 5 g. of phosphotungstic acid. The precipitate formed was redissolved by warming in a water-bath and allowed to crystallise at room temperature for 2 days. It was then separated by centrifuging, washed with a solution of 2·5 g. phosphotungstic acid and 3·5 g. hydrochloric acid in 100 cc. water, and finally dissolved in a strong solution of sodium hydroxide. Nitrogen was determined on an aliquot. The basic material thus separated from the dicarboxylic acid precipitate contained 0·6 % of the total N. Allowing for this the value for the dicarboxylic acid-N was 28·0 %.

Precipitation of the bases. The alcoholic mother-liquor and washings from the calcium dicarboxylates were made neutral with acetic acid, evaporated *in vacuo* to remove all alcohol and made up to a volume of 50 cc. The precipitation

with phosphotungstic acid was now carried out according to the Van Slyke procedure, except that the precipitate was separated by centrifuging instead of by filtration. It was dissolved in a strong solution of sodium hydroxide and nitrogen was determined on an aliquot.

The mother-liquor and washings were treated as in the usual Van Slyke analysis and both total N and amino-N determined.

Effect of the precipitation of the dicarboxylic acids on the "basic" N. Parallel with the analysis of gliadin already described another determination was made in which the normal Van Slyke procedure was followed, the bases being precipitated directly after the removal of ammonia. The value thus obtained was 10·7 %. No solubility correction was applied in view of the recent work of Thimann [1930] on the bases. In the first analysis, the basic material precipitated from the dicarboxylic acid fraction was 0·6 % (in terms of total N) and 10·2 % from the remaining solution, thus giving a total value of 10·8 %. The agreement is considered sufficiently good for present purposes.

Similar experiments were made with certain other proteins, the basic N being determined in one case directly and in the other after precipitation of the dicarboxylic acids. It was found that varying amounts of basic material were removed with the dicarboxylic acids, a maximum of 2·2 % of the total N being found with caseinogen. Experience suggests that the governing factor is not the percentage of the bases present in the protein, but the physical condition of the precipitate and that by adding the alcohol in small quantities with vigorous shaking the amount of basic impurities could be reduced to a minimum. In all cases the bases were quantitatively recovered by precipitating the dicarboxylic acid solution itself with phosphotungstic acid.

Examination of the small precipitate obtained on adding large volumes of alcohol to the solution from which the calcium dicarboxylates had been precipitated as above. 10 g. of gliadin were hydrolysed and dicarboxylic acids separated as before. To the mother-liquid thus obtained an equal volume of alcohol was added and the solution kept overnight. The precipitate was filtered off and dissolved in the minimal amount of concentrated hydrochloric acid; the solution was saturated with hydrogen chloride gas and kept in the ice-chest. No separation of glutamic acid hydrochloride was observed. A similar small precipitate obtained from another 10 g. of gliadin hydrolysate was treated with copper oxide. No insoluble copper salt was obtained. These results confirm those of Foreman with caseinogen.

Effect of dissolving the dicarboxylic acid fraction and reprecipitating with alcohol. The precipitate obtained from gliadin with calcium hydroxide and alcohol, as already described, which contained 28·0 % of the total nitrogen was dissolved in about 15 cc. of water and reprecipitated by the addition of alcohol as before. The nitrogen content of the second precipitate thus obtained was 27·7 % of the total protein-N, showing that the amount of impurities removed by a second precipitation was inconsiderable.

Analysis of various proteins.

Of the proteins analysed the following were prepared in the laboratory according to the usual methods: gliadin, zein, edestin (from hemp-seed), haemoglobin and serum-globulin (from horse-blood). B.D.H. preparations of the following proteins were used: caseinogen, egg-albumin, pepsin. The leaf proteins analysed were the original preparations of these proteins made by Prof. Chibnall to whom I am indebted for their gift.

The nitrogen contents of the preparations used (calculated on an ash- and moisture-free basis) are given in column 3 of Table I. Though complete analyses were carried out on most of the proteins examined only the figures relevant to the present purpose are quoted. The amide-N figures are included in every case on account of their significance with regard to the relationship that is usually assumed to exist between the ammonia and the dicarboxylic acids resulting from the acid hydrolysis of proteins.

Table I. *Analysis of certain proteins.*

All figures given are in percentages of total protein nitrogen.
(A, present analyses. B, other workers*.)

							Basic N				
							A				
								Indirectly precipitated			
	Protein	Total N	Amide-N A	Amide-N B	Dicarboxyl c acid-N A	Dicarboxyl c acid-N B	Directly pre-cipitated	In dicarb-oxylic acid fraction	In main solution	Total	B
Proteins recently analysed for their dicarboxylic acid content:											
1	Gliadin	17·3	25·7	25·52 24·61 26·13 25·9	28·0	26·79[1]	10·7	0·61	10·2	10·8	7·26 5·61 12·91 10·97
2	Edestin	18·5	10·2	9·99	15·0	15·6[1]	35·1	1·84	32·63	34·5	38·15 31·7
3	Caseinogen	14·6	10·3	10·31 10·43	15·4	14·8[2]	23·1	2·20	20·45	22·7	24·12
4	Egg-albumin	14·9	8·8	8·65	14·7	12·3[1]	24·2	0·84	23·09	23·9	21·27
5	Zein	16·0	19·6	20·75	17·5	18·56[3]	—	0·56	2·90	3·5	3·1
Other proteins:											
6	Haemoglobin	16·9	5·6	5·24 6·37	9·4	—	—	0·44	28·80	29·2	31·3
7	Serum-globulin	15·9	8·1	—	13·1	—	—	1·21	22·77	24·0	25·25
8	Pepsin	13·4	3·6	—	12·3	—	—	0·35	21·2	21·6	—
Leaf proteins:											
9	Spinacin	16·3	—	6·93[4]	17·7						
10	*Zea Mays*	14·4	—	7·44[4]	21·9						
11	Runner bean	15·9	—	5·69[4]	20·8						
12	Alfalfa	15·7	—	5·5[4]	20·1						

[1] Jones and Moeller [1928]. [2] Foreman [1914]. [3] Dakin [1923]. [4] Chibnall and Grover [1926].

* Except where specific reference numbers are given all data in columns B are taken from Plimmer [1917], or Hoffmann and Gortner [1925].

Discussion.

The values for the dicarboxylic acids for the proteins 1–5 given by the method now described are in close agreement with those of previous workers who have used modern methods for the direct isolation of the acids themselves, and show that a good insight into the dicarboxylic acid content of proteins can be obtained by this simple addition to the usual procedure of Van Slyke. The values for the basic N determined directly and indirectly also show that the modification introduces no appreciable error in the determination of this group.

It is interesting to re-examine in the light of the analytical results now recorded the close agreement found by Osborne, Leavenworth and Brautlecht [1908] between the amide-N of proteins and their content of dicarboxylic acids. Assuming that one carboxyl of the acids was present as an amide group they calculated the proportion of ammonia which should be liberated by acid hydrolysis and compared this with the amount actually found. In Table II their results, recalculated to conform with the data given in Table I, are compared with those now available. Whereas Osborne found the agreement was fair in most cases, in others, especially the prolamins, the divergence was wide. The discovery of hydroxyglutamic acid and improvements in the methods for estimating glutamic and aspartic acids have so increased the total dicarboxylic acid content that the prolamins now constitute the only group which shows anything approaching agreement; the others all have an excess of dicarboxylic acids. This is very marked in the case of the leaf proteins which exhibit, as a class, the same regularity in dicarboxylic acid content as Chibnall and Grover [1926] found for the bases. Such a close agreement in amino-acid composition is not found in any class of seed proteins.

Table II.

Protein	Dicarboxylic N (Osborne)	Dicarboxylic N (from Table I)	Amide-N (Osborne)
Edestin	9·68	15·6	9·99
Glutenin	13·33	16·4[1]	18·87
Caseinogen... ...	7·28	15·4	8·49
Ovalbumin... ...	7·12	14·7	8·66
Gliadin	20·47	28·0	24·61
Zein...	11·69	18·56	18·44
Hordein	20·1	23·6[2]	23·31
Prolamin from Ragi	—	22·3[3]	21·4[3]

[1] Damodaran [1931]. [2] Kleinschmidt [1907]. [3] Damodaran, this paper.

In view of the large margin of error in calculating the values for the different amino-acids in the basic precipitate it is tentatively suggested that the determination of the following groups would give a reliable distribution of nitrogen for the characterisation of proteins:

(i) Amide-N.
(ii) Humin-N.
(iii) Dicarboxylic acid-N.
(iv) Basic N.

(v) Arginine-N, determined directly according to Plimmer and Rosedale [1925, 2].

(vi) Non-amino-N.

(vii) Monoaminomonocarboxylic acid-N.

NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

Ragi (*Eleusine coracana*) forms the staple cereal food of the people in some of the drier parts of India. It was hoped to make a study of all the proteins in this seed, but unfortunately only one, a prolamin, could be prepared in a state of purity.

The average protein content of the seed was 6·8 % (N × 6·35) and it consisted of a globulin, a prolamin, and a glutenin. From the finely ground ether-extracted seed a 9 % solution of sodium chloride (found by trial to be the optimal strength) extracted 29·2 % of the total N. The residue after being washed free from sodium chloride and air-dried was exhaustively extracted with warm 73 % alcohol to dissolve out the prolamin. 40·2 % of the total N was removed by this treatment. The residual material was finally treated with 0·4 % sodium hydroxide which dissolved a further 12·6 % of the N. 82·8 % of the seed-N was thus accounted for. Attempts to isolate the globulin by dialysis of the sodium chloride extract and of the glutenin by precipitation from the sodium hydroxide solution gave products which were impure and contained only about 12 % N. Variations in the method, such as treatment with takadiastase to remove carbohydrate, and extraction with alcoholic potash as recommended by Jones and Csonka [1927] were tried without success.

Experimental.

It was found that the quickest and most satisfactory method for preparing the prolamin was to extract the finely ground seed with boiling 73 % alcohol for 1 hour, filter and evaporate *in vacuo* to small volume. By preventing the alcoholic strength from going much below 70 %, either by the addition of further portions of the extract or of alcohol, the extract could be concentrated to a thick syrup without causing any denaturation of the protein. The syrupy solution so obtained was poured with vigorous stirring, while still warm, into about 20 volumes of acetone, when the protein was precipitated as a fine powder.

The substance, after filtering at the pump and drying in air, was purified by redissolving in a small volume of warm 73 % alcohol and reprecipitating with acetone as before. The air-dried powder contained 6·1 % moisture, 0·9 % ash, and moisture- and ash-free, 16·4 % N. A portion was redissolved in alcohol and again precipitated with acetone; but the nitrogen content was not

thereby increased. Tests for sulphur were negative. The isoelectric point of the protein, determined approximately by dialysing a suspension in distilled water in a cellophane bag for 48 hours, and measuring electrometrically the minimal p_H attained, gave a value between p_H 4·8 and p_H 5·0.

The prolamin was analysed by the method outlined in this paper.

Nitrogen distribution.

	% total N
Humin-N	0·8
Amide-N	21·4
Dicarboxylic acid-N...	22·5
Basic N	5·8
Arginine-N	2·2
Monoamino-monocarboxylic acid-N	44·1
Non-amino-N	5·1

It will be seen that the protein has an amino-acid composition characteristic of the prolamins. The amide-N and dicarboxylic acid-N are high, and in close agreement, while the basic N is relatively low [Damodaran, 1931].

My thanks are due to Prof. A. C. Chibnall for the interest he has taken in this work and for much valuable advice.

REFERENCES.

Andersen and Roed-Muller (1915). *Biochem. Z.* **73**, 326.
Chibnall and Grover (1926). *Biochem. J.* **20**, 113.
Dakin (1923). *Z. physiol. Chem.* **130**, 159.
Damodaran (1931). *Biochem. J.* **25**, 190.
Foreman (1914). *Biochem. J.* **8**, 463.
Gortner and Sandstrøm (1925). *J. Amer. Chem. Soc.* **47**, 1663.
Hoffmann and Gortner (1925). Colloid Symposium Monograph, **2**, 209.
Jones and Csonka (1927). *J. Biol. Chem.* **74**, 427.
—— and Moeller (1928). *J. Biol. Chem.* **79**, 429.
Kingston and Schryver (1924). *Biochem. J.* **18**, 1070.
Kleinschmidt (1907). *Z. physiol. Chem.* **54**, 110.
Onslow (1930). Plant biochemistry. (Cambridge.)
Osborne, Leavenworth and Brautlecht (1908). *Amer. J. Physiol.* **23**, 180.
Plimmer (1916). *Biochem. J.* **10**, 115.
—— (1917). Chemical constitution of the proteins, p. 97. (Longmans, Green and Co., London.)
—— and Rosedale (1925, 1). *Biochem. J.* **19**, 1004.
—— —— (1925, 2). *Biochem. J.* **19**, 1021.
Thimann (1930). *Biochem. J.* **24**, 368.
Wada (1930). *Biochem. Z.* **224**, 420.

CCXXIX. VITAMIN A AND CAROTENE.

VIII. HIGH POTENCY VITAMIN A CONCENTRATES.

By THOMAS MOORE.

From the Dunn Nutritional Laboratory, Cambridge.

(*Received November 2nd, 1931.*)

In a previous communication [Moore, 1931, 1] experiments were described which involved the feeding of large amounts of carotene, as red palm oil, to albino rats. Since by this means the vitamin A content of the untreated liver oils could often be made to exceed that of high quality cod-liver oil concentrates it was realised that a good opportunity was afforded to carry the concentration of the vitamin beyond the limits previously attainable.

Preliminary results, communicated at a meeting of the British Association [Moore, 1931, 2], indicated that activities represented by a minimal rat dose of about 0·001 mg., or by colour values 10 times that of carotene, could be attained in concentrates prepared from the liver oils of either rats or pigs which had received liberal amounts of red palm oil. Moreover since oils of widely divergent potency were found to give final concentrates having colour values approaching a common maximum it was considered that these preparations must consist substantially of the vitamin, or alternatively that the vitamin must be associated with other substances in remarkably constant proportion.

In apparent conflict with the view expressed above Karrer, Klussmann and Euler [1931] reported the preparation of a concentrate from the liver oil of *Hippoglossus hippoglossus*, which was at first claimed to display chromogenic activity no less than 130 times that of carotene, equivalent to 10,000 "C.L.O. units." Although a correction has now been made to adjust the ratio from 130 to 13 when pure β-carotene is taken as the standard [Karrer, Morf and Schöpp, 1931; Euler and Karrer, 1931] it still might appear, from the extremely high value given in C.L.O. units, that this concentrate was considerably more potent than those prepared by the present writer[1]. To throw further light on this point Prof. von Euler kindly agreed to supply samples of highly potent fish-liver oils, in order that concentrates derived from them might be compared directly with those derived from rat- and pig-liver oils. The results to be described tend to indicate that the fish oils, rat- and pig-liver oils all yield

[1] Karrer, Morf and Schopp [1931] find that geronic acid is a common decomposition product of carotene and vitamin A concentrates when treated with ozone. From the amount of geronic acid semicarbazone derived from the *Hippoglossus* concentrate they calculate that 50–80 % of actual vitamin A was present.

preparations of approximately equal activity when the same processes of concentration are applied.

Experimental.

Rat-liver oils. Samples of rat-liver oil were obtained from two single animals and from a group of 34 animals. In all cases 15 % of red palm oil was administered for some months before killing, but apart from this essential feature the diets given differed in detail. Thus sample 1 was obtained from 34 rats which received a diet composed of skimmed milk powder 20 %, rice starch 60 %, red palm oil 15 %, salts 5 %; while sample 2 was derived from a single rat which received "light white casein" 20 %, rice starch 60 %, red palm oil 15 %, dried yeast (additional) 15 %. Sample 3 differed decidedly in being obtained from an animal which was exposed to deficiency of the vitamin B complex in connection with another experiment [Dann and Moore, 1931]. The liver oils were obtained by the method previously described, involving digestion of the tissues by 5 % aqueous potassium hydroxide followed by extraction with ether.

Pig-liver oils. The use of pigs was made possible through the kindness of Lever Brothers, Ltd., who undertook the feeding of the animals and also arranged for the extraction of the liver oils. The red palm oil was given as an addendum to the normal diets of the pigs in amounts up to 12 ozs. per pig per day, the feeding being commenced at the "porker" stage (live weight approximately 40 kg.) and continued until the animals reached maturity as bacon pigs. To extract the oils the livers were steamed, minced, pressed free from water, dried and solvent-extracted.

Fish-liver oils. Of the oils supplied by Prof. von Euler one was a sole-liver oil and the remaining two turbot-liver oils. The blue values determined by Miss Rydbom before dispatch were given as 347, 930 and 1200 c.l.o. units respectively.

Concentration of the vitamin.

The methods employed in the concentration of the vitamin were the same in all cases and involved no novel feature. The oils were saponified by a brief boiling with alcoholic potassium hydroxide, ether being used for the extraction of the unsaponifiable matter. The vitamin fraction was usually separated from the bulk of crystalline material by extraction with cold methyl alcohol, the last traces of sterols were then removed by digitonin. The final concentrates were compared by the antimony trichloride method applied in the way previously described. The results obtained are shown in Table I. It should be noted that the values given for the percentage of unsaponifiable matter and "final concentrate" are those actually observed, no allowance having been made for possible losses in manipulation.

$SbCl_3$ absorption band.

Observations by means of a pocket spectroscope revealed the prominent absorption band at 620$m\mu$ typical of vitamin A concentrates.

Biological tests.

As a biological check upon the colorimetric data rat growth tests were carried out in the case of the concentrate from the rat-liver oil No. 2. The basal diet used consisted of Glaxo caseinogen 20 %, rice starch 75 %, salts 5 %, dried yeast (additional) 7·5 %, radiostol (vitamin D) 1 drop per rat daily. The test

Table I.

Description and source	B.U. per g. liver	B.U. per mg. untreated oil	% total unsap. matter	% final concentrate	B.U. per mg. final concentrate	% deviation from average
Rat-liver oil No. 1 from 34 rats	1,600	90	16	3·5*	2100	12
Rat-liver oil No. 2	7,000	450	24	18·2	1900	21
Rat-liver oil No. 3 from "B free" rat	13,000	1000	47	32·6	2300	4
Pig-liver oil No. 1	—	70	17	2·8	2100	12
Pig-liver oil No. 2	—	90	13	3·3	2600	8
Sole-liver oil	—	100	7	3·3	2700	12
Turbot-liver oil No. 1	—	400	14	9·3	3000	25
Turbot-liver oil No. 2	—	450	17·5	13·7	2400	0
				Average ...	2400	

* Digitonin process omitted.

Note. Values given in B.U. per mg. can be converted to the scale of blue values advised by the Pharmacopœia Commission by multiplying by 18·2.

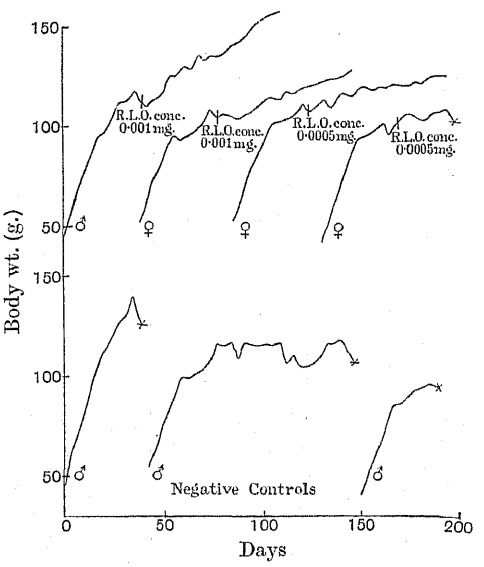


Fig. 1. Biological check of vitamin A activity carried out on concentrate from rat-liver oil (R.L.O.) No. 2.

doses were made up in coconut oil, which possesses a great advantage over many natural oils in not interfering with the antimony trichloride reaction when it is desired to check the stability of the solutions [Norris and Church, 1930]. From Fig. 1 it will be seen that slow growth was restored by doses of

0·001 mg. of concentrate. Of two animals receiving 0·0005 mg. one showed very slow growth while the other died.

Discussion.

Constancy of the final concentration. The outstanding feature of the above results lies in the constancy of the vitamin A activity, as judged by the $SbCl_3$ reaction, attained in the various concentrates. Not only did the values given by the rat- and pig-liver oil preparations as a class approximate closely to those given by the fish-liver oil preparations, but in each class oils of widely different potencies gave final concentrates of almost equal activities. As an extreme illustration of this point attention may be directed to rat-liver concentrates Nos. 1 and 3 which from original colour values of 90 and 1000 B.U. per mg. respectively gave concentrates of almost equal activity. The original blue values of any oil, indeed, could be taken as an indication of the quantity of concentrate that could be obtained, and not of the final strength of the concentrate[1].

Degree of concentration attained. The average $SbCl_3$ value of 2400 B.U. per mg. (Pharmacopœia blue value 45,000) given by the concentrates was about 12 times that given by the specimen of carotene recently supplied for experiments on the international standardisation of vitamin A and 2400 times greater than the specimen of cod-liver oil supplied on the same occasion. In view of the possibility of errors arising through small modifications in technique, personal factors in matching shades, and above all through variations and misunderstandings in the mode of expression of results, it would perhaps be inadvisable to attempt a comprehensive comparison of these figures with those given by Euler and Karrer for their *Hippoglossus* concentrate. A thorough study of such data as are available, however, suggests that this preparation was of the same order of activity as those at present under investigation, and this conclusion is borne out by the closely concordant results given by the *Hippoglossus* and rat-liver oil concentrates in biological tests. It seems possible, from a consideration both of the colour data and of the additional stages of freezing and adsorption adopted in preparing the *Hippoglossus* concentrate, that an activity some 70 % greater than the average value found in the present experiments was attained. Such a superiority would imply that the simplest explanation of the constant activity of the final concentrates obtained in the present experiments—that they contained a predominant proportion of vitamin A—could not hold. It would become necessary to assume that the vitamin in its most active sources is associated with a remarkably constant proportion of non-chromogenic material possessing similar solubilities.

The conversion of carotene. The above work permits further light to be shed on the phenomena accompanying the conversion of carotene to vitamin A.

[1] This statement must for the present be confined to oils similar to those under investigation. With oils of lower colour value, such as ordinary cod-liver oils, it does not seem possible to attain concentrates of such high activity, at least by the simple methods employed in the present work.

In view of the blue values given by the concentrates it must be implied that conversion is accompanied by at least a 12-fold increase in chromogenic activity. At the same time it is not necessary to infer that these spectroscopically different colorations bear a common relation to biological activity. Thus, although Karrer, Morf and Schöpp [1931] state that their *Hippoglossus* concentrate possessed 10 times the biological activity of carotene, this conclusion is not clear from the evidence presented by Euler and Karrer [1931]. A careful comparison of the biological activity of the most active concentrates against carotene in parallel experiments seems to be demanded, and is now in progress in this laboratory.

Another point of interest lies in the extreme change in the solubility in methyl alcohol from the insoluble carotene to the almost miscible vitamin A. This matter has received previous mention [Moore, 1930] but deserves emphasis in view of the recent work of Angus *et al.* [1931] who find that vitamin D is much more freely soluble in this solvent than the pro-vitamin ergosterol. When it is also recalled that vitamin E is likewise readily soluble in methyl alcohol [Olcott and Mattill, 1931] it becomes apparent that the fat-soluble vitamins as a class are sharply characterised in this respect.

Summary.

Vitamin A concentrates prepared from the liver oils of rats and pigs which had received diets containing large amounts of carotene, as red palm oil, were compared with concentrates derived from turbot- and sole-liver oils. Although the initial blue values of the oils varied widely little difference could be detected in the activities of the final concentrates, which approached an average value of 2400 B.U. per mg. (Pharmacopœia colour value 45,000) in the antimony trichloride test, corresponding to a minimal dose of about 0·001 mg. in rat growth experiments. These results suggest either that the concentrates must have consisted substantially of actual vitamin A, or alternatively that the vitamin must have been associated with other substances of similar solubility properties in remarkably constant proportion.

My thanks are due to Dr L. J. Harris for his valuable criticism. The care of the experimental animals was in the hands of Mr A. W. Davies.

REFERENCES.

Angus, Askew, Bourdillon, Bruce, Callow, Fischmann, Philpot and Webster (1931). *Proc. Roy. Soc. Lond.* B **108**, 340.

Dann and Moore (1931). *Biochem. J.* **25**, 914.

Euler and Karrer (1931). *Helv. Chim. Acta*, **14**, 1040.

Karrer, Klussmann and Euler (1931). *Arkiv. Kem., Min., Geol.* **10**, No. 16.

—— Morf and Schöpp (1931). *Helv. Chim Acta*, **14**, 1036.

Moore (1930). *Biochem. J.* **24**, 692.

—— (1931, 1). *Biochem. J.* **25**, 275.

—— (1931, 2). Report of the British Association.

Norris and Church (1930). *J. Biol. Chem.* **85**, 477.

Olcott and Mattill (1931). *J. Biol. Chem.* **93**, 59.

CCXXX. A STUDY OF THE FACTORS INFLUENCING THE CONCENTRATION OF COMPLEMENT IN THE BLOOD.

By THOMAS WILLIAM BRYANT OSBORN.

From the Department of Biochemistry, Oxford University.

(*Received July 23rd, 1931.*)

The present work was commenced with the idea of determining what changes take place in the development of vitamin A deficiency which renders animals susceptible to bacterial infection. It was felt that this would throw new light on the problem of general immunity, since it presented an easy method of lowering and raising the immunity at will.

Of all known factors in general immunity complement is the one which is most widespread, and, therefore, immediately suggested itself for investigation. It is not possible to say dogmatically that a low blood-complement necessitates a low degree of resistance, as there is not enough evidence to prove this. Much of the evidence up to the present points to this conclusion, however, and many workers in this field seem to assume it [see Cadham, 1926]. Moore [1919] tested this assumption by direct experiment and his results showed that it was true for the animals and conditions he used. Working with a number of guinea-pigs low in complement, he found that, though their specific immunity was not significantly below normal, their general immunity was.

It was decided to determine the complement concentration in the sera from vitamin A-deficient rats at various stages of the deficiency, and to compare this with that of normals. Any differences produced by the administration of cod-liver oil were to be investigated.

Several other problems of a similar nature presented themselves during the course of the work. Indications were found that the age of the animal had an influence, and that the ingestion of food produced a change. Observations on these two points were carried out. Huntermuller [1930] has made the interesting observation that doses of ultra-violet radiation raised the complement in man. A series of observations, therefore, was made on the effect of irradiation by means of a mercury vapour lamp on the blood-complement of rats.

Method of estimating complement.

As it was required to make a large number of estimations on the small amount of blood to be obtained from the tail of a rat any method requiring more than one-tenth of a cc. of serum was excluded. In addition to this, an

attempt was made to avoid the inaccuracy of the "serial tube" methods. After considerable experiment, a modification of Eagle's method [1929] was devised, and the writer has found it to be satisfactory in the course of some three thousand estimations.

Sheep's blood, defibrinated by whipping with glass rods and filtered through muslin soaked in saline, was used as a source of the corpuscles. It could be kept in a cold store for periods not exceeding 3 days. The corpuscles were deposited by centrifuging and were washed 3 times with 20 vols. of normal saline (0·9 % NaCl). They were then shaken with a volume of saline sufficient to make the total volume equal to 10 times the original volume of the blood, thus giving an approximately 5 % suspension of corpuscles, assuming the volume of corpuscles equivalent to about 50 % of original blood. The sensitising agent was glycerinated "anti-sheep corpuscle" serum having 1000 units per cc. (Burroughs and Wellcome).

0·01 cc. of this serum—containing 10 units of complement—was made up to 1 cc. with normal saline, and this was added to 1 cc. of the 5 % suspension of corpuscles.

Before constructing the standard "haemolysis time-complement concentration" curve, it was necessary to measure the complement concentration of a volume of serum by the direct serial tube method. The approximate concentration was determined by adding graded volumes of serum, from 0·0025 to 0·025 cc. in each case made up to 0·05 cc. with saline, to 10 tubes, each containing 0·05 cc. of the suspension of sensitised corpuscles. The tubes were placed in a water-bath at 37° for 15 minutes, and the minimal volume of serum necessary for complete haemolysis was determined. This volume was determined more precisely by repeating the operation, adding volumes of serum covering a smaller range. The liquids were measured with a capillary pipette of 0·05 cc. capacity, graduated into 10 divisions. This was used with a small rubber teat. It was found to be important to empty the pipette at about the same rate each time. The haemolysis mixture was made in agglutination tubes of the Dreyer pattern.

Varying volumes of the serum, whose complement concentration had been determined accurately by the direct serial tube method, were mixed with 0·025 cc. of the 2·5 % suspension of corpuscles in agglutination tubes, and the total volume was made up to 0·2 cc. The time for haemolysis was measured in a thermostat at 37°. The amount of complement used, as calculated from the volume of serum used and the known complement concentration of the serum, was plotted against the time for complete haemolysis (Fig. 1).

In constructing the curve a sample of guinea-pig serum and a sample of human serum were used. In making the estimation on a sample of rat blood, it was the practice to use two or more different volumes of serum, and as the different points fell consistently on the curve, it is clear that the curve is applicable to rat's as well as to human and guinea-pig's sera.

It is the custom to express the concentration of complement as the minimal

volume of serum sufficient to haemolyse a definite volume of corpuscles. A more convenient expression is the reciprocal of this volume. For the purpose of this research, it was decided to adopt, as the unit concentration of complement, that amount which is just sufficient to haemolyse an equal volume of a 2·5 % suspension of sheep's corpuscles, sensitised with 10 units of anti-sheep corpuscle serum, in 15 minutes at 37°. The volume of corpuscle suspension used divided by the minimal volume of serum necessary for complete haemolysis gives the complement concentration of the serum in these empirical units.

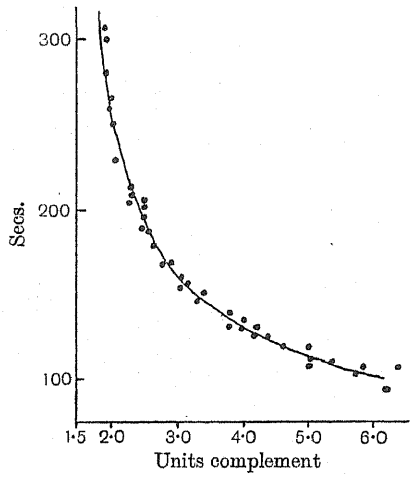


Fig. 1. Standard haemolysis time-complement concentration curve.

In order to estimate the complement concentration of an unknown serum, a volume of this serum is mixed with corpuscles as described, the total volume being made up to 0·2 cc. with normal saline. The time for complete haemolysis at 37° is measured. By reference to the curve, the number of units of complement to give this result is ascertained, and, by multiplication, the number of units in 0·025 cc. of serum—the volume of corpuscles used—may be calculated. This figure represents the complement concentration of the serum.

Certain points of technique require special attention. The end-point of complete haemolysis is rather indefinite. To get as good conditions for the observation as possible, a thermostat with glass sides is used, and a brightly illuminated black and white background is placed behind the tubes.

It was found that, in using these small volumes, any visible roughness of the glass was liable to cause errors, probably due to adsorption effects. It is therefore necessary to select very carefully the tubes to be used. Since any deposit left by tap-water is detrimental to the result, the tubes should be rinsed with distilled water several times before being dried. Both in diluting the serum and in adding the corpuscles, thorough mixing is necessary. This can best be done, in small volumes, by blowing one or two small bubbles into the liquid with the pipette and rocking the tubes so that the bubbles act as mixers.

It will be seen from the curve that the points below 120 seconds are rather scattered. Any haemolysis which takes longer than about 240 seconds has an especially vague end-point. Therefore, for accurate work, only the portion of the curve lying between 120 and 240 seconds should be used.

Rous and Turner [1915] advise particular care in the preparation of the washed corpuscles. Their method of drawing the blood into a weak solution of citrate and gelatin may possess advantages, but, as the method of using whipped blood was proving satisfactory, it was not thought advisable to change it. Brooks [1919] used a buffered Ringer solution instead of saline. He used a bicarbonate buffer, which, of course, has the disadvantage of slowly losing carbon dioxide and so becoming more alkaline. It is difficult to see why he chose to work at p_H 7·8, when, if he were going to use a buffer, he might easily have worked at the p_H of the circulating blood.

An idea of the accuracy of the method may be gained from the closeness of the points to the curve in Fig. 1. It has been found that the error is relatively greater in samples of serum poor in complement. This may be due to the relatively greater amounts of serum-proteins present. An analysis of the last 400 duplicate estimations made by the method, using only that part of the standard curve between 120 and 240 seconds, showed the error to be as follows:

	Below 5 units %	5–15 units %	Above 15 units %
Mean deviation (½ difference of duplicate estimations)	7·6	3·2	2·7
Maximum observed deviation	25	10	7·5

One large source of error was that different samples of sheep's blood differed considerably in the ease with which their corpuscles were haemolysed. This made it necessary to assay each sample. Valley and McAlpine [1928] state that a good way to preserve complement in serum is to keep it away from corpuscles in an atmosphere of carbon dioxide. By preparing a standard sample of serum and keeping it under these conditions, and by titrating each sample of corpuscles against it, a curve for the depreciation of complement in this serum was obtained. A correction was applied to each of those samples of serum which showed a deviation from the curve.

To obtain a sample of blood from the rat, the tail was warmed in normal saline at 45° for 1 minute; the end was cut with a pair of scissors and a few drops were squeezed out on to a waxed plate. A piece of glass tubing had one end drawn out into a capillary. The blood was drawn up into the tube through the capillary portion before it had time to clot, and the end of the capillary was sealed in a flame. When a clot had formed, a fine glass needle was passed between it and the walls of the tube to prevent the clot sticking to the glass. The tube was stoppered and allowed to stand overnight. No appreciable change took place if the sample was kept in a cold store for 48 hours.

INFLUENCE OF DIETARY DEFICIENCY.

Early experiment. Two groups of animals which were at hand offered an opportunity of testing whether animals on a poor basal diet differed from animals which had taken a liberal supplement of vitamin-containing foods. Rats 420 to 426 had been fed on mixed corn and brown bread from the time of weaning. This is a poor diet, though it is not absolutely deficient in any vitamin. Rats 325 to 328 had received a diet of purified caseinogen, starch and salts, with 3 % cod-liver oil. During part of this time they had been deprived of one of the constituents of the vitamin B complex, but, before the time of the commencement of the experiment, they had been brought to a normal condition by the addition of 6 % marmite to their diet. These animals were put on a diet of corn and brown bread. Another litter of rats was treated in a similar manner. Four, A 18 to A 21, were given corn and brown bread alone, and two, A 17 and A 22, had a liberal supply of green stuff, milk and cod-liver oil added to this diet.

All the animals were given 1 drop of "radiostol" per week throughout the experiment to ensure a good supply of vitamin D. According to the specification of the makers, this is equivalent to about 40 rat units per day, so that even allowing for some depreciation the animals should have been taking an excess of vitamin D.

Each sample was taken in the morning after a fast of 16 hours. The results are given in Table I. It will be seen that the animals which had received a rich

Table I.

A, 3 days after 10 drops of cod-liver oil (0·33 cc.). B, 4 days after 10 drops of cod-liver oil.

Basal diet *plus* excess cod-liver oil, milk and greens		Basal diet. Mixed corn and brown bread	
Rat No.	Complement	Rat No.	Complement
325	15·0	420	4·6
	12·7		6·1
	14·0	421	1·9
	13·0		6·4
326	12·2	422	1·8
	17·1		10·3 A
	14·7	423	4·7
	10·7		8·7
327	10·7		15·6 B
	16·9	424	10·7
328	13·5	425	6·4
	15·4	426	6·6
A 17	7·3	A 18	3·4
	20·8		3·1
A 22	9·0	A 19	3·4
	16·8		3·0
		A 20	3·1
			3·1
		A 21	8·6
			6·2

diet had much more complement than the others. It was significant that rats 422 and 423 had a high complement a few days after a dose of 10 drops of cod-liver oil.

The basal diet seemed to have no absolute deficiency, and it was not clear what it was in the supplements which caused the difference in complement concentrations. It was decided to perform an experiment to compare complement in animals fed on a diet deficient in vitamin A with that in others having cod-liver oil added to the diet. Before this was done it was considered to be advisable to investigate in more detail the influence of age, the ingestion of food and illumination.

The effect of the age of the animal.

In the course of previous investigations indications were found that there was a tendency towards a low complement in young rats. This does not fit in with the findings of workers on human beings [see Goldner, 1929; Gunn, 1914; Moro, 1902]. Kolmer, Matsunani and Trist [1919] state that the blood of young guinea-pigs is more frequently low in complement than that of adults, but give no figures in support of this statement.

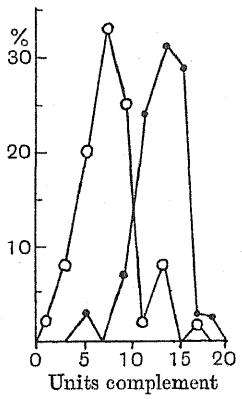


Fig. 2. Percentage distribution of complement values in young and adult rats.
○—○ Young rats.
•—• Normal adult rats.

Complement titrations have been performed on blood from 43 young animals between the age of weaning and 4 weeks from weaning, the animals being weaned between the ages of 3 and 5 weeks according to their weight. Almost all the samples were found to be poor in complement as compared with normal adult animals, whether the samples were taken in the morning after a 16-hour fast, or in the afternoon 3 hours after feeding (Table II and the distribution curves of Fig. 2). In making the comparison, only those figures for young rats are used which are directly comparable with the adult normals, *i.e.* only estimations on blood samples taken in the morning after 16 hours of fasting. The young rats include animals both on a deficient diet and on a rich diet.

The results show that there is a marked tendency for the complement to be low in the blood of young rats and, therefore, only rats of more than 4 weeks from weaning should be used in these experiments.

The effect of ingestion of food.

In the course of the main investigation, indications were received that, in some cases, the ingestion of food caused a change in complement. Cadham [1926], Hadjapoulos and Burbank [1928] and Gunn [1914] mention that a slight rise in complement may be noticed during the day, but they do not correlate this with any digestive activity. Kolmer, Matsunani and Trist [1919]

Table II. *Comparison of complement concentrations in the blood of young and adult rats.*

	Young rats		Normal adult rats	
	No. in range	% in range	No. in range	% in range
0– 2 units	1	2	0	0
2– 4 „	3	8	0	0
4– 6 „	8	20	2	3
6– 8 „	13	33	0	0
8–10 „	10	25	4	7
10–12 „	1	2	14	24
12–14 „	3	8	18	31
14–16 „	0	0	16	29
16–18 „	1	2	2	3
18–20 „	0	0	2	3

	Young rats	Normal adult rats
Percentage below 2 units	2	0
„ „ 4 „	10	0
„ „ 6 „	30	3
„ „ 8 „	63	3
„ „ 10 „	88	10
„ „ 12 „	90	34
„ „ 14 „	98	65
„ „ 16 „	98	94
„ „ 18 „	100	97
„ „ 20 „	100	100

state that blood taken after a 24-hour fast is richer in complement than blood taken immediately after a meal.

A short investigation was made to ascertain whether any definite changes occurred. Out of 23 experiments, 11 showed no change, 12 showed definite changes, comprising a definite fall, a rise and then a fall, or a steady rise (Table III). The basal diet referred to is the fat-free vitamin A-deficient diet described later.

Ehrlich [1906] has suggested that the mechanism for the destruction of bacteria in the blood is the same as that for the utilisation of food material carried by the blood. On this hypothesis, it would be expected that, under certain conditions, a change in some of the agents of immunity might be found during digestion of food. This change might also be expected from a consideration of the suggestion of Walker [1905–06] that the enzymes themselves use a "complement."

Sufficient data have been obtained to show that in some circumstances and in certain animals a change does take place. What the conditions are under which a change occurs, and what regulates the type of change, are problems which will be investigated at a future date.

Effects of irradiation with a mercury vapour lamp.

Huntermuller [1930] investigated the effects of ultra-violet light in winter on 18 human beings, most of whom were normal. His conclusion is that a good, but not excessive, dose causes an immediate slight rise in complement, then a

Table III. *Changes in complement concentration after ingestion of food.*

Rat 289. Basal diet *plus* 10 drops olive oil		Food intake g.	Rat 432. Basal diet		Food intake g.	Remarks
Initial	16·7	—	Initial	16·2	—	No change outside limits of accuracy of the method. Eleven results of this type
1 hour	16·7	6	1 hour	15·6	8	
2 hours	15·4	5	2 hours	15·8	0	
3 hours	15·4	1	3 hours	15·4	0	
4 hours	15·4	2		Total food...	8	
5 hours	16·7	1				
	Total food...	15				
Rat 369. Basal diet			Rat 331. Basal diet			
Initial	11·2	—	Initial	13·6	—	Definite immediate fall. Five results of this type
1 hour	10·0	2	1 hour	10·0	3	
2 hours	9·2	2	2 hours	10·6	2	
3½ hours	10·2	2	3 hours	11·6	1	
5 hours	8·3	1	4 hours	12·0	1	
	Total food...	7	5 hours	14·3	0	
				Total food...	7	
Rat 432. Basal diet			Rat 423. Basal diet			
Initial	8·7	—	Initial	4·8	—	Rise followed by a fall. Four results of this type
¾ hour	9·7	10	1 hour	13·0	10	
1½ hours	6·9	0	2 hours	5·6	2	
3 hours	6·0	2	3 hours	5·3	0	
	Total food...	12	5 hours	5·3	0	
				Total food...	12	
Rat 327. Basal diet			Rat 332. Basal diet			
Initial	14·0	—	Initial	4·0	—	Steady rise. Three results of this type
1 hour	15·4	6	1 hour	4·0	10	
2½ hours	17·0	0	2 hours	11·0	0	
4 hours	19·0	0	3 hours	11·3	3	
	Total food...	6	4 hours	11·5	2	
			5 hours	13·5	0	
				Total food...	15	

fall, and, in 2 or 3 hours, a steady rise reaching a maximum in about 20 hours, then a gradual fall back to the initial value in the course of several days. Unfortunately he quotes no figures to show the extent of the rise, and he does not state whether he finds that it occurs invariably or only in a majority of cases.

In investigating the effects of irradiation on the complement, a mercury vapour lamp was used which produced a definite erythema on human skin at a distance of 6 inches in 2 minutes. The animals were placed in their cages, all food having been removed, at a distance of 20 inches from the lamp. Since the cages had a metal grid bottom, there was no possibility of an effect from the ingestion of irradiated sawdust from the tray of the cage. The experimental animals were controlled by animals, if not from the same litter, of the same age and having an identical feeding history. The controls were kept in cages

apart from the experimental animals, but they were kept under the same conditions during the experiment, being carried into the lamp room with the experimental animals to control any effect of handling or of temperature change. In some cases, a control experiment was performed on the same animal, either before or subsequent to the experimental observation.

Twenty-eight animals were used in this work. Twenty-six experimental and 18 control observations were carried out. In most cases an observation consisted of taking a sample at 10 a.m., immediately before the irradiation, another at 12.30 p.m., and a third at 6 p.m. In a few experiments longer intervals were allowed between sampling. The exposure varied from 2 to 4 minutes at 20 inches.

A typical example of the results obtained is given in Table IV. Of the 26 observations, 6 gave a definite rise, 3 gave a doubtful rise and 17 gave no significant rise above the controls. The important fact, from the point of view of the main thesis of this work, is, that none of the 10 rats on the vitamin A-deficient diet submitted to the light showed a significant rise in complement. The data therefore show that, while the mercury vapour lamp causes a rise in complement in rats on some occasions—a fact which corresponds with Huntermuller's

Table IV. *Examples of the effect of irradiation with a mercury vapour lamp on complement.*

		Complement			
		10 a.m.	12.30 p.m.	6 p.m.	Remarks
Rat A 17	3 mins. irradiation	3·2	8·1	10·9	Rise
Rat A 22	Control	3·8	3·2	4·0	
Rat A 18	3 mins. irradiation	1·8	3·3	3·5	No rise
Rat A 19	„ „	1·9	2·8	5·4	? rise
Rat A 20	Control	1·7	1·7	2·9	
Rat A 21	„	1·7	2·3	3·3	
Rat 381	3 mins. irradiation	7·8	10·0	13·1	Rise
Rat 420	„ „	14·5	11·4	16·3	No rise
Rat 479	„ „	7·8	5·8	10·3	No rise
Rat 421	„ „	11·0	9·0	13·4	

observations on human beings—it does not do so invariably. This conclusion is further strengthened by the observation that 8 vitamin A-deficient rats and 7 young rats, which were daily exposed to the radiation for 2 to 3 minutes, all had a complement figure below 8 units on at least one occasion.

Effect of a diet deficient in vitamin A.

The main experiments which follow were controlled by rigidly defining the ages of the animals and the time of estimation of the complement after feeding. Further, the animals were all kept in a darkened room. No rat was used in this experiment under 4 weeks from weaning.

Twenty-four animals from 5 litters were put on a diet of:

	g.
Purified rice starch	70
"Glaxo" vitamin A-free caseinogen	20
McCollum's salt mixture	5
Agar	2
Marmite	6

The marmite was increased to 10 g. during the course of the experiment, as on theoretical grounds 6 % is barely sufficient for the maintenance of an adequate supply of the vitamin B complex. Some of the animals, as controls, were given a supply of the fat-soluble vitamins either as cod-liver oil or as "radiostoleum." All the others were given 1 drop "radiostol" per week to ensure a supply of vitamin D.

As the absence of fats from the diet might have had an effect on the complement level, another group of animals was given

	g.
Rice starch	50
Hardened cotton-seed oil	20
McCollum's salt mixture	5
"Glaxo" vitamin A-free caseinogen	20
Marmite	10

As before, some of these animals were kept as controls and were given 5 drops of cod-liver oil per day, and the others were given 1 drop of "radiostol" per week.

Blood samples were taken from these rats at intervals of about a week. In the beginning and during most of the course of the experiment, the samples were drawn in the morning, after a 16-hour fast. During the latter part of the experiment a few samples were taken at 3.30 p.m., 3 hours after new food had been put in the cages.

In all, 36 animals were used on this part of the work. Twenty-four were placed on the vitamin A-deficient diet, and 12 were given an adequate supply of the vitamin in the form of cod-liver oil or "radiostoleum," and showed no abnormalities in growth or symptoms associated with vitamin A deficiency. An analysis of the figures obtained showed that, while the complement only fell below 10 units in 2 out of 12 normal, it fell below that level in 22 out of the 24 vitamin A-deficient animals.

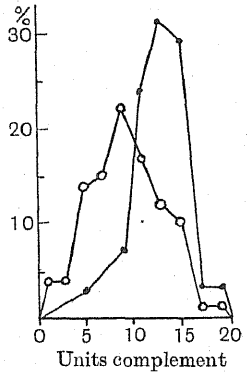


Fig. 3. Percentage distribution of complement values.
•—• Normal rats.
○—○ Deficient rats.

A statistical analysis of the figures is shown graphically in Fig. 3, and in tabular form in Table V. Each point in the first figure represents a reading between the nearest whole numbers. In the second figure the percentages of the total number of readings within certain ranges are plotted for normal and deficient animals. Only readings obtained after a 16-hour fast have been reckoned; those taken at 3 hours after feeding gave a similar result.

Table V. *Distribution of complement estimations for rats on basal diet deficient in vitamin A and the same supplemented with cod-liver oil.*

	Rats on basal diet		Rats on basal diet *plus* cod-liver oil	
	No. in range	% in range	No. in range	% in range
0– 2 units	3	4	0	0
2– 4 ,,	3	4	0	0
4– 6 ,,	11	14	2	3
6– 8 ,,	12	15	0	0
8–10 ,,	18	22	4	7
10–12 ,,	14	17	14	24
12–14 ,,	10	12	18	31
14–16 ,,	8	10	16	29
16–18 ,,	1	1	2	3
18–20 ,,	1	1	2	3

	Rats on basal diet	Rats on basal diet *plus* cod-liver oil
Percentage below 2 units	4	0
,, ,, 4 ,,	8	0
,, ,, 6 ,,	22	3
,, ,, 8 ,,	37	3
,, ,, 10 ,,	59	10
,, ,, 12 ,,	76	34
,, ,, 14 ,,	88	65
,, ,, 16 ,,	98	94
,, ,, 18 ,,	99	97
,, ,, 20 ,,	100	100

It may be concluded, from the results obtained that there is a marked tendency towards low blood-complement in animals deficient in vitamin A or some other factor, other than vitamin D, occurring in liver oils. In the deficient animals marked fluctuations in complement were found which may be due, in part, to the infections to which such deficient animals are susceptible, and, in part, to whatever it is that causes fluctuations from day to day in normal rats and human beings. The fact that animals which are well supplied with liver oil are occasionally deficient in complement, indicates that the presence of liver oils in the diet is not the only condition which must be fulfilled for the maintenance of a high complement. It has been a common experience in bacteriological laboratories that the complement of guinea-pig's serum, high in the summer, may be low in the winter without any alteration in the diet. It is significant that the few instances in which a low blood-complement has been found in rats receiving cod-liver oil have all occurred in winter months.

Discussion.

Irregularity of results. One of the most noticeable features of the results recorded in this work, and, indeed, of any other work which has been done involving the determination of haemolytic complement, is the irregularity of the results. This must be due to the existence of factors influencing complement which have not yet been discovered. This variability makes it necessary

to obtain a large number of observations, and to describe any changes as "tendencies" instead of as definite changes.

In considering these results it should be borne in mind that weight curves of rats deprived of vitamin A are seldom even, and that there is very little constancy about the reaction of deficient animals to cod-liver oil. The day-to-day variation of complement in human beings and rats is difficult to explain. Some of the reported variations have undoubtedly been experimental artefacts due to a variation in the corpuscles used in the haemolysis system. Even when a correction is made for this by the method described for assaying the susceptibility of corpuscles, there is still a large variation.

Relation to other deficiencies. Although lowered resistance is not as prominent a symptom of vitamin B, C, or D deficiency as it is of vitamin A deficiency, there is good support for the view that it is found in these conditions. Werkman, Nelson and Fulmer [1924] showed that guinea-pigs suffering from scurvy were less resistant to bacterial infection than normals, and Findlay [1925] confirmed this. Werkman [1923] showed that rats and pigeons lacking vitamin B were less immune to attacks from *B. anthracis* and the *Pneumococcus* than normal animals. Findlay and Maclean [1925] found that blood from animals deficient in vitamin B or vitamin D had a lowered bactericidal power. Smith and Wason [1923] confirmed this for vitamin D-deficient animals.

The question of whether the lowering of complement in animals fed on a diet deficient in vitamin A is specific, or whether it is common to all vitamin deficiencies, has had some attention. In a cursory investigation, consisting of 8 observations on the blood of 4 rats deficient in vitamin B_1, the author found no significant change in complement. This, however, should by no means be accepted as conclusive. Zilva [1919] found no change in guinea-pigs on a scorbutic diet. Koch and Smith [1924], on the other hand, found a slight rise during the development of scurvy. Smith and Wason [1923] found no change in rats fed on a rachitic diet.

Inanition due to uncomplicated starvation is generally thought to lower resistance to bacterial infection. Koch and Smith [1924] found a drop in complementary power during starvation, but Zilva [1919] found no loss of complement in guinea-pigs after they had been kept on a quantitatively restricted diet for 6 months.

It is certain that there is a lowered resistance in dietary disturbances other than vitamin A deficiency. The evidence is against these being due to diminished complement activity, but this should not be considered as proven.

The action of ultra-violet radiation. The action of ultra-violet light on the body is a much disputed subject. Many people have claimed that it raises the resistance of the body to infection. Colebrook, Eidenow and Hill [1924] showed that it raised the bactericidal power of the blood. This increase was of such a transitory nature that many people were unwilling to attach much importance to it. Colebrook [1929] undertook an extended research and failed to find that ultra-violet light improved the general health in any way, while Hill and Lawrie

[1931] reaffirmed the claim of many that it did. The increased resistance produced by ultra-violet light is thought to be quite apart from its well-known power of producing vitamin D.

The finding of Huntermuller, that ultra-violet light increases complement, is a big advance and may be the means by which many of the actions of ultra-violet light will be explained. The finding that this increase does not occur in all cases of laboratory animals does not detract from the importance of Huntermuller's discovery. These exceptions are of interest in this research because they make it unlikely that ultra-violet light and cod-liver oil cause a rise in complement through the same agency. The evidence for this is that none of the vitamin A-deficient animals submitted to ultra-violet light reacted with a definite rise in complement, and a number of animals which were daily exposed to irradiation had low complements during periods of vitamin A deficiency.

Summary.

1. A method of estimating haemolytic complement is described, which has greater accuracy and uses smaller quantities of blood than the serial tube method now in general use.

2. A unit of complement is defined, which is the reciprocal of the minimal volume of serum sufficient to haemolyse a given volume of sensitised blood corpuscle suspension.

3. Young weaned rats tend to have a lower complement than adult animals.

4. The ingestion of food may cause a variation in the blood-complement of rats.

5. Irradiation with a mercury vapour lamp sometimes causes a rise in complement (Huntermuller), but enough evidence has been obtained to show that this effect is not general.

6. The haemolytic complement tends to be lower in the blood of rats fed on a diet deficient in vitamin A than in the blood of controls receiving cod-liver oil.

The writer is indebted to Prof. Peters for his interest and advice during this research, to Dr V. Reader for many suggestions in regard to the animal experiments, to Dr R. A. Fisher for a statistical examination of the results, to Dr K. H. Coward for advice in vitamin work and to Miss Kempson for her care of the animals. The research was carried out while the writer was the holder of a Rhodes scholarship.

REFERENCES.

Brooks (1919). *J. Med. Res.* **41**, 399.
Cadham (1926). *Can. Med. Assoc. J.* **16**, 352.
Colebrook (1929). *Med. Res. Council Reports*, No. 131.
—— Eidenow and Hill (1924). *Brit. J. Exp. Path.* **5**, 54.
Eagle (1929). *J. Gen. Physiol.* **12**, 821.
Ehrlich (1906). Studies in immunity. (John Wiley and Sons, New York.)
Findlay (1925). *Brit. J. Exp. Path.* **6**, 16.
—— and Maclean (1925). *Biochem. J.* **16**, 574.
Goldner (1929). *Deutsch. med. Woch.* **55**, 390.
Gunn (1914). *J. Path. Bact.* **19**, 155.
Hadjapoulos and Burbank (1928). *J. Lab. Clin. Med.* **14**, 131.
Hill and Lawrie (1931). *Lancet*, i, 182.
Huntermuller (1930). *Strahlentherapie*, **25**, 489.
Koch and Smith (1924). *Proc. Soc. Exp. Biol. Med.* **21**, 366.
Kolmer, Matsunani and Trist (1919). *Amer. J. Syph.* **3**, 407.
Moore (1919). *J. Immunol.* **4**, 425.
Moro (1902). *Jahrb. Kinderheilk.* **55**, 396.
Rous and Turner (1915). *Proc. Soc. Exp. Biol. Med.* **12**, 122.
Smith and Wason (1923). *J. Immunol.* **8**, 195.
Valley and McAlpine (1928). *J. Immunol.* **15**, 313.
Walker (1905–06). *J. Physiol.* **33**, *Proc.* xxi.
Werkman (1923). *J. Inf. Dis.* **32**, 247, 255, 263.
—— Nelson and Fulmer (1924). *J. Inf. Dis.* **34**, 447.
Zilva (1919). *Biochem. J.* **13**, 172.

CCXXXI. ON THE FUNCTION OF TORULIN. AN *IN VITRO* EFFECT OF ANTINEURITIC VITAMIN CONCENTRATES.

By NICOLAI GAVRILESCU and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(*Received October 31st, 1931.*)

Recently, the authors [1931, 1] have shown that there is a lowered oxygen uptake[1] as compared with the normal in pieces of minced avitaminous pigeon's brain tissue suspended in glucose-phosphate solution[2]. The change was most marked in the optic lobes. Cure of the pigeon was accompanied by an increase in oxygen uptake, at a time when no increase in body weight occurred. This was proof that a change had occurred in the tissue as the result of introducing vitamin into the body, but not that the increase was due to the addition of vitamin as such to the tissue. Two things suggest that the changes in avitaminosis were due to the local lack of vitamin: (1) that the changes in the brain tended to be localised, and (2) that the most rapid cures of head retraction observed in this Department were induced by the injection of torulin into the cranium; in some cases indeed cure of symptoms has occurred in 30 minutes. Striking as this evidence is, it is not conclusive. The missing entity in the tissue, indicated by the localised chemical lesion, might be some hormone either made from the vitamin and brought to the brain in the circulation, or secreted as the result of a stimulus of vitamin. It has in fact been stated by Cramer [1930] that vitamin B extracts cause a rapid liberation of adrenaline from the suprarenal gland. Such an explanation might also apply to the injection experiments. There are two ways of settling the question; either it might be proved that there is less vitamin B_1 in the optic lobes, *etc.* than normal, which is not practical with the pigeon test owing to the small amounts of tissue involved,

[1] We prefer to use the expression "oxygen uptake" which exactly expresses the observations and is at present non-committal as to their meaning.

[2] About the time that the earlier results were communicated to the Biochemical Society, a paper appeared by Abderhalden and Vlassopoulos [1931] which we did not see until recently. The authors claim to have established a lowering of oxygen uptake in liver, brain and muscle, restored when the birds are dosed with yeast extracts. We believe that the experiments are open to certain objections in the case of the brain tissue which is the only one upon which we have worked. Rather low figures are obtained, very likely owing to the large pieces of tissue used (0·5 g.). Our own preliminary results previously reported, show not much difference in Ringer phosphate solution for oxygen uptake of cerebrum. No attempt seems to have been made to differentiate between different parts of the brain.

or it might be shown that the oxygen uptake *in vitro* is increased by the addition of vitamin concentrates. We believe that this proof has now been obtained, and present the evidence for it below [1931, 2]. If confirmed by further work, it will be the first instance in which an effect of a vitamin has been demonstrated upon a tissue *in vitro*.

Experimental.

The tissue. In all cases we have used birds in the standard condition of opisthotonus induced by our usual methods. Sometimes the cases had been used previously for vitamin tests. We noticed no essential difference between birds which were fresh avitaminous cases and those which had a renewed attack after previous vitamin dosing. There appears to be no point in recording details of the birds used. The effects of vitamin were observed upon the mixed optic lobes and lower parts of the brain (optic lobes/rest) and the cerebrum respectively in the presence of air and of oxygen. Doses of vitamin ranging from 0·01 to 2·0 pigeon-doses have been used, the most satisfactory amount appearing to be 0·1–0·05 pigeon-dose per 3·0 cc. of fluid, containing 0·08–0·10 g. of tissue. Since animal tissues usually contain amounts of vitamin B_1 of the order of 1–2 pigeon-doses per g. of tissue, the dose appears to be optimal if all adsorbed by the tissue. This is very likely to be the case at p_H 7·4.

The tissue was very finely minced as before with a bone spatula and thoroughly mixed, the mixing being continued before the withdrawal of each sample for a bottle. Samples were placed in the bottles in irregular order to avoid any possible influence of drying. Where possible determinations were made in triplicate, but the amounts of tissue are not large. A total of about 0·9 g. of minced tissue can be obtained from the cerebrum, and about 0·4 g. from the mixed optic lobes/rest. Cerebellum was not included in the study as no change in this tissue was previously observed.

The measurements. The methods used were essentially the same as previously employed; the glucose-phosphate buffered solution was made up as before; the precautions of Dixon and Elliott [1930] as to rate of shaking and absorption of CO_2 were observed as previously. The constants were checked by direct determinations under the conditions of the experiments at 38°. In the first series air was used in the bottles and in the later oxygen. The bottles were evacuated to a pressure of 710 mm. Hg before filling with oxygen and washed out once with the gas. About 20 minutes elapsed from the guillotining of the bird to placing the apparatus in the warm bath when air was used, and about 30 minutes with oxygen. It was our practice to shake for 12 minutes before taking the first reading, and to make subsequent readings every 10 minutes for 60 minutes. The results were calculated as an average for the hour of observation. With the cerebrum, the rate of oxygen uptake remained fairly constant though there was a tendency to "balling" of the tissue in some cases. This was noticeable as a lowered oxygen uptake in the first periods. In some cases allowance could be made for this by a comparison of subsequent periods

(Exps. 99, 114 and 117 have been so corrected). Both in cerebrum and optic lobes/rest, comparison of results with and without vitamin for the last 40 minutes of the periods of shaking gave substantially the same results. With the optic lobes/rest the rate of oxygen uptake fell off throughout the period of observation to an amount which varied in different experiments from 33 to 50 % of the initial rate.

The errors of the observations cannot be computed exactly. Those due to the apparatus alone are not larger than 1 %. As we have worked with a mixed tissue, we have included in the Tables all those results which could not be explained by us as due to experimental error. It appears from the figures that our results are subject in the extreme to variations of the order of $\pm$ 10 %, though many duplicate estimations are much better than this. Doubtless several of the discrepancies are due to undetected experimental error; others are probably due to unequal adsorption of vitamin upon the pieces of tissue.

Vitamin solutions. Torulin (yeast B_1) concentrates of different purity were used. The crude preparation A consisted of a 50 % alcohol concentrate of activity about 3·0 mg./pigeon-dose (1·5 B_1 units) [for details see Carter, Kinnersley and Peters, 1930][1]. Preparation B was less impure, and was an X concentrate, prepared initially from brewer's yeast, of activity 0·05 mg./day. This constitutes a purer source of vitamin B_1 than the Y concentrates, so far as other factors in the B-complex are concerned. Preparation C was one of our purest concentrates of activity 12γ/day [see Kinnersley and Peters, 1930, 2]. The solutions were freshly prepared; two drops of water were added to the alcoholic solution (about 0·1 cc.), the alcohol present was removed by heating on the water-bath with continuous shaking; a trace of sulphuric acid (N/20) was then added, and the heating continued for 2 minutes. This was necessary in most cases in order to remove the last traces of barium with which it is our custom to store many of the preparations. The torulin solution was diluted to about 0·5 cc., a minimal amount of phenol red added, and the whole carefully neutralised to p_H 7·4; water was added to make the vitamin addition 0·1 cc. in each bottle. In all the later experiments, a control tube was treated similarly as to sulphuric acid, phenol red and soda and added to the control brain sample.

In the experiments in which alkali-treated vitamin was used, the procedure was the same up to the alcohol stage. After this, however, the sample was divided into two parts. One of these was neutralised approximately, the requisite amount of NaOH added and the sample heated in the water-bath in a test-tube immersed in the boiling water, with a funnel to prevent excessive concentration. After treatment with NaOH, a similar amount of cold NaOH was added to the other sample of active vitamin; the two were then neutralised in a similar way with addition of the necessary amount of phenol red. In this way the effects of excess of salt were controlled. The additions of torulin were made immediately before placing the apparatus in the bath, to avoid possible inactivation.

[1] 1 pigeon-dose = approx. 15 mg. Jansen acid clay $\equiv$ 1·5 vitamin units.

Results.

The results are given in Table I, and will be considered in detail below. Before doing so mention must be made of the effect of oxygen.

Table I. *Protocols. Oxygen uptake, mm.³/g./hr.*

Air. Avitaminous birds at H.R. stage.

(*a*) Control and vitamin.

	Cerebrum				Optic lobe and rest (mixed)			
Exp.	Control	Vitamin	Dose added	Diff. % average	Control	Vitamin	Dose added	Diff. % average
84	1230 1190	1245 1225	2·0 A	+2	—	—	—	—
99	1100 1090	1090 1140	0·1 A	+2	790 950 940	880 940 1050	0·1 A	+7
100	—	—	—	—	810 880 960	840 1015 1030	0·1 A	+7
106	925 —	1290 1270	0·1 A	—	930 930	1120 1050	0·06 A	+16
107	1155 1220 1285	1190 1240 1380	0·06 A	+4	870 940	890 1060	0·06 A	+8
108	1080 1130 1130	1150 1180 1240	0·1 A	+7	—	1130 980	0·7 A	—
109	1080 1125 1180	1140 1290 1350	0·1 A	+11	810 880	950 1070	0·1 A	+20
110	840 —	1090 —	0·1 A	—	745 765 795	835 890 900	0·1 A	+13

(*b*) Control, alkali-treated and active vitamin.

	(1)	(2)	(3)		Diff. %		(1)				Diff. %	
Exp.	Control	Alkalised vitamin	Active vitamin	P.D.	1/2	2/3	Control	Alkalised vitamin	Active vitamin	P.D.	1/2	2/3
112	1080 1120	1070 1110 1130	—	0·1 A	Nil	—	870 920	855 950	—	0·1 A	+1	—
114	1030 1170	910 1080	1214 —	0·1 B	−10	—	—	760 815	850 865	0·1 B	—	+9

In presence of oxygen.

(*c*) Comparison of oxygen and air, together with vitamin and oxygen.

	Cerebrum					Optic lobes and rest (mixed)				
Exp.	Air	(ii) O_2	(iii) O_2+vit.	Dose	ii/iii Diff. %	Air	O_2	O_2+vit.	Dose	ii/iii Diff. %
117	980 995	1220 —	1470 —	0·1 A	[+19]	810	780 920	930 1000	0·1 A	+15
118	955 1015	1330 1360	1520 1580	0·1 A	+15	810	905 965	920 1150	0·1 A	+10
119	1070 1150	1470 1550	1645 1700	0·1 A	+10	1135	1040 1190	1240 1275	0·1 A	+13
120	890 975	1250 1320	1220 1470	0·09 B	+5	1030	1140 1170	1330 1460	0·08 B	+21
123	1120 1130	1200 1500	1480 1620	0·11 B	+15	805	1020 1030	—	—	—

Table I (*cont.*)

(*d*) Active, alkali-treated vitamin and controls in presence of oxygen.

	Cerebrum						Optic lobes and rest (mixed)						
	(1)	(2)	(3)		Diff. %		(1)	(2)	(3)		Diff. %		
Exp.	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/2	2/3	Control	Alkalised vitamin	Active vitamin	Dose	1/2	2/3	Remarks
124	—	1530 1720 1810	1600 1660 1870	0·1 B	—	+2	—	745 972	1090 1130	0·1 B	—	+28	2 *N* NaOH 20 min.
125	—	1330 1495 1660	1720 1730 1750	0·07 B	—	+16	—	1005 1180	1030 1250	0·07 B	—	+5	0·5 *N* NaOH 45 min.
126	1130 1210 1280	—	1190 1220 1325	0·01 B	+2	—	752 760	—	810 915	0·01 B	+13	—	
127	—	1470 1790	1500 1750 1790	0·05 B	—	+3	—	1210 1350	1340 —	0·05 B	—	Nil	0·5 *N* NaOH 30 min.
128	—	1610 1670 1690	1690 1720 1820	0·05 B	—	+5	—	1130 1260	995 1210	0·01 B	−8	—	0·5 *N* NaOH 30 min.

Active, alkali-treated vitamin, *etc.* (*continued*).

	Cerebrum						Optic lobes and rest (mixed)						
	(1)	(2)	(3)		Diff. %		(1)	(2)	(3)		Diff. %		
Exp.	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/3	2/3	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/3	2/3	Remarks
129	—	1510 1550 1580	1340 1350 1720	0·05 B	—	−9	—	1070 1045	1345 1380	0·05 B	—	+30	0·5 *N* NaOH 30 min.
130	1705 1825	1710 1760	1920 1935	0·01 B	+10	+11	—	1120 1160	1080 1160	0·01 B	—	+2	0·5 *N* NaOH 30 min.
131	1175 1185	1330 1400	1455 1500	0·05 B	+25	+7	—	925 930	980 1280	0·05 B	—	+23	2 *N* NaOH 60 min.
133	1190 1270	1390 1420	1440 1490	0·10 B	+19	+4	—	890 915	1040 1070	0·10 B	—	+20	2 *N* NaOH 60 min.
138	1550 —	— —	1560 1550	0·1 C	—	Nil	1125 1180	—	980 1135	0·01 C	−10	—	
139	1340 —	— —	1410 1460	0·1 B	+7	—	1030 895	—	875 1110	0·01 B	+3	—	
150	1200 1240	—	1300 1380 1425	0·10 B	+17	—	910 940	—	900 975	0·05 B	+1	—	
151	1410 1490 1510	—	1490 1520 1600	0·10 C	+14	—	805 920	—	970 1140	0·1 C	+16	—	
153	1280 1380	1190 1220	1220 1260	0·05 C	−7	−4	—	1240 1330	1390 1570	0·05 C	—	+15	2 *N* NaOH 30 min.
154	1640 1700	1500 1560	1530 —	0·05 C	−9	Nil	—	1170 1280	1400 1400	0·05 C	—	+14	2 *N* NaOH 30 min.
164	—	—	—	—	—	—	1150 1225	—	1262 1304 1340	0·1 C	+10	—	Brains of 2 birds mixed
165	—	—	—	—	—	—	1120 1120 1190 1175	—	1204 1340 1220 1200	0·1 C	+6	—	Brains of 2 birds mixed
184	1302 1430	—	1240 1425	0·1 C	−25	—	1202 1225	—	1280 1382	0·1 C	+10	—	
185	—	—	—	0·1 C	—	—	990 1130 1152	—	1240 1310	0·1 C	+16	—	

Comparison of the results with oxygen and air respectively.

It was stated previously that the uptake of oxygen could be increased by performing the experiments with oxygen in the bottle instead of air, but that our general conclusions were not influenced by this. In Table II we have recorded the average results from several recent experiments together with those from our previous paper. It will be seen that the new ones are quite in agreement with the old. The conclusion that there is lowered oxygen uptake in glucose-phosphate solutions in the avitaminous brains is made more certain by these results. The increase in oxygen uptake for oxygen as against air with

Table II. *Comparison of oxygen uptake in presence of air and oxygen.*

(*a*) Different birds. mm.^{3}/g./hour*.

Tissue		Air	Limits	Oxygen	Limits	Increase
Cerebrum	N.	**1315**	(1000–1600)	**1810**	(1470–2220)	+38 %
	Av.	**1030**	(880–1210)	**1341**	(980–1670)	+32 %
Optic lobes/rest	N.	**1100**	(960–1280)	**1480**	(1300–1600)	+38 %
	Av.	**880**	(805–1135)	**1055**	(980–1670)	+16 %

N. = Normal. Av. = Avitaminous.

(*b*) Comparisons upon tissue from same bird (avitaminous).

	Cerebrum		OL/LP	
	Air	O_2	Air	O_2
(1)	990	1220	810	850
(2)	980	1340	810	930
(3)	1110	1510	1135	1120
(4)	880	1280	1030	1150
(5)	1050	1350	805	1030

* 1000 mm.^{3}/g./hr. = Q_{O_2} (Warburg) 5·0 approx.

normal brains is rather variable, amounting on the average to + 38 % for cerebrum and + 32 % for the rest. This would be interpreted easily upon the usual view that the presence of oxygen activated underlying pieces of the tissue not otherwise reached at the pressure of oxygen in air, if it were not for the results with the avitaminous tissue. With the avitaminous cerebrum, increased oxygen pressure increased the uptake by 30 %. With the avitaminous mixed optic lobes and lower parts, however, the uptake was not increased on the average more than some 20 %, and in several cases which are given in abstract in Table II a comparison of the tissue with and without oxygen shows no difference. Since there is no apparent difference in the state of division of the tissue for normal and avitaminous brains, doubt is thrown upon the simple explanation that the increases observed in these minced brain samples are due merely to increased penetration of oxygen. As previously mentioned, at the conclusion of the experiment the tissue is mostly in the form of a fine suspension. The matter merits further attention. In the series of experiments which follow, the effects of vitamin concentrates have been followed both in the presence of air and of oxygen; rather similar results have been obtained with each.

Effects of vitamin concentrates.

In the protocols given in Table I, we have given the experiments as they were obtained, and have included all those in which no obvious experimental error could be detected. In this way the whole evidence upon which conclusions are based is before the reader. It will be recalled that previously we reached the conclusion that there are differences between the cerebrum and the rest of the brain in avitaminosis. Kinnersley and Peters [1930, 1] advanced the view that the changes in lactic acid metabolism commenced in the lower parts of the brain and gradually spread to the cerebrum, and in our previous paper we concluded that the two parts of the brain were not always equally affected. In this work too, we have found marked differences between the behaviour of the two parts, and are therefore considering them below as separate tissues.

Mixed optic lobes and the rest.

A casual glance at the protocols for this part of the work shows that in spite of variations the general trend of the results is towards an increase in the presence of vitamin. The results are shown diagrammatically in Fig. 1. Out of 19 experiments, 6 in air, and 13 in oxygen, 10 show a clear increase for the vitamin-treated tissue in the sense that there is no overlap in the figures; 7 of these 10 are in the presence of oxygen[1]. We have examined and excluded the possibility that some particular use of the same bottles for the same parts of the experiment might have led to the result, as also that the order in which the tissue was transferred to the bottles might produce the effects found. The other possibility that the results might be the consequence of the method of determining constants has also been eliminated by finding these directly at 38°, which led to a slight constant correction. The problem therefore narrows down to the following question; what are the chances that we have made favourable selections in these 10 experiments? To take a concrete case, Exp. 119, we might suppose that the possible errors in sampling this particular tissue lay between 1000 and 1300, and that by chance we had selected our samples to give the apparent increase for vitamin. The matter is one of statistics, and the probability against this favourable selection is very great. The mean percentage difference is 10 % approx. It can be calculated that a mean percentage difference of 4·8 % would be likely to occur by chance only once in 100 times. We feel therefore that the results constitute proof that the addition of vitamin concentrates increases the oxygen uptake. The effect is catalytic: where the preparations B and C have been used, a larger weight of oxygen is taken up by the vitamin-treated tissue than the weight of solid added. As an example with the purest C, 0·5γ added to 3·0 cc. of the solution increased the average O_2 uptake by 15γ oxygen. Controls showed that no increase took place in absence

[1] The remainder show an average increase except Exp. 138, which cannot be explained and was performed with 1/100 pigeon-dose of vitamin, a rather small amount.

of tissue. The average increase of 12 % is about half that of the lowered oxygen uptake previously recorded.

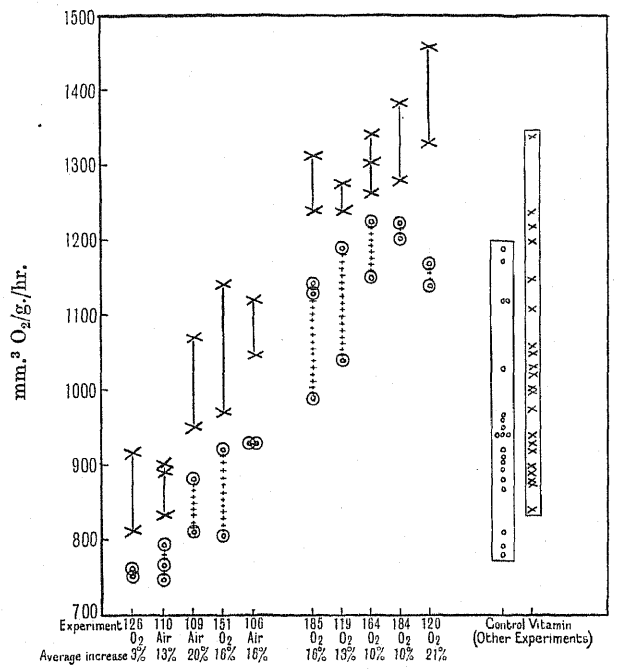


Fig. 1. Chart showing results for mixed optic lobes/rest with and without vitamin. × + vitamin concentrates, O control. Ordinates O_2 mm.³/g./hr. Experiments arranged along abscissae in increasing order of basal oxygen uptake. Other results for Exps. 99, 100, 107, 117, 118, 150, 165, 139, *etc.*, grouped together and shown on right of chart.

The next question, whether the increase is due to the vitamin itself or to some other base in the concentrates, cannot be settled absolutely in the absence of pure vitamin B_1. Concentrates of quite different purity give the same result. Treatment with alkali is known to inactivate vitamin B_1. It has been shown previously that 1 hour's heating at the temperature of the boiling water-bath with 0·5 *N* NaOH will inactivate a concentrate of 0·06 mg. activity. This probably varies for the preparation, and requires reinvestigation [Kinnersley and Peters, 1928]. In the experiments below, it appeared from pigeon tests that 30 minutes' heating at the temperature of the boiling water-bath with approx. 0·5 *N* NaOH was not enough to inactivate preparation B, but that 30 minutes with 2*N* NaOH was enough. We have compared the effect of alkali-treated concentrates with untreated controls, containing a similar amount of salt. In Table III the results of this treatment have been collected for the oxygen experiments and arranged in order of alkali treatment. 0·5 *N* NaOH for 30 minutes seems to be marginal, whereas 2*N* NaOH for 30 minutes seems to be sufficient to inactivate the factor concerned. Such a treatment was found by animal tests to reduce the activity to less than 10 % of the original. The

Table III. *Oxygen uptake (mm.³/g./hr.) for mixed optic lobes/rest with active and alkali-treated vitamin B concentrates.*

Exp. No.	Dose added	Pre-paration	Vitamin: Alkali-treated	Vitamin: Active	Difference	Alkali treatment at 100° approx.
127	0·05	B	1210, 1350	1340, —	[? nil]	0·5 *N* 30 min.
129	0·05	B	1070, 1045	1345, 1380	+30 %	0·5 *N* 30 „
125	0·07	B	1005, 1180	1030, 1250	+5 %	0·5 *N* 45 „
124	0·10	B	745, 972	1090, 1130	+28 %	2*N* 20 „
153	0·05	C	1240, 1330	1390, 1570	+15 %	2*N* 30 „
154	0·05	C	1170, 1280	1400, 1400	+14 %	2*N* 30 „
131	0·05	B	925, 930	980, 1280	[+23 %]	2*N* 60 „
133	0·10	B	890, 915	1040, 1070	+20 %	2*N* 60 „

Note. The above experiments in oxygen. Exp. 114 (air) gives a similar result. Exps. 128 and 130 not included, in which heating was for 30 min. with 0·5 *N* NaOH, and only 1/100 dose used.

inactivating effect of alkali therefore is roughly the same upon vitamin B_1 and the factor considered. Here again 0·01 pigeon-dose seems to be marginal. Taken together with the fact that our purest concentrates show the effect, we have reached the best proof at present available that the oxidation factor concerned is vitamin B_1. The necessary concentration so far as can be judged from this limited series of experiments lies between 0·1 and 0·01 pigeon-dose, the latter being marginal. With preparation C, this means a dilution in 3·0 cc. of about 1 in 30,000,000.

The cerebrum.

We have mentioned previously our conviction that the condition in this tissue is different. To the eye, the minces from the two tissues appear quite dissimilar. It has been steadily forced upon us during the course of this work that the behaviour towards vitamin concentrates is also different, a conclusion quite at variance with our preconceptions. The effects of different concentrates do not appear to be identical, nor does the inactivation follow the same course, though there is ample proof that the addition of certain concentrates improves the uptake of oxygen.

Out of 6 completed experiments in air, 2 showed no overlap between the figures (Nos. 99 and 108). The remainder only showed a small increase upon the average. For experiments in oxygen, out of 13 completed experiments, 6 (Nos. 118, 119, 130, 131, 133, 150) showed an increase such that there was no overlap in the duplicate observations (in some cases triplicate). Of the remainder, 4 showed increases upon averages of different degree. Three gave a

decrease. This we cannot explain at present, but it is noteworthy that the two significantly negative results were obtained with the purest preparation (90 A). There is a definite tendency (see also 138) for this preparation to be less effective than the others in increasing oxygen uptake in the cerebrum. Before discussing the possible meaning of this, we can consider the results for activated and inactivated vitamin. In the experiments with each bird, the vitamin solution was the same throughout; hence any variations in the inactivation should affect both cerebrum and the rest equally. It is noticeable that there are cases in which the difference for active and inactive vitamin is definite for the optic lobes, *etc.*, but is not so for the cerebrum. This is illustrated by Exps. 124, 129, 133, 153 and 154. It appears to be more difficult to inactivate the preparation for cerebrum than for the rest. From each set of experiments therefore we reach the conclusion that in the cerebrum the lowered oxygen uptake is due to causes other than vitamin B_1 deficiency alone. It is possible that the vitamin is unstable in the presence of factors sometimes associated with the cerebral tissue, but in view of the apparent difficulty of inactivating the tissue for the cerebral factor, a more attractive hypothesis is that the cerebral tissue is suffering from an additional deficiency of some other factor. This would be consistent with the very irregular action of our purest preparation, which for instance contains very little vitamin B_4. Such speculations must be rather random in the absence of work upon the more obvious points which require an answer. Much might be learnt for instance from a comparison of the effect of vitamin upon the tissue in the presence and absence of glucose. This should settle how far the effect was exerted upon the residual oxygen uptake. Our present conclusion must be that vitamin B_1 is less consistently effective in catalysing an increased oxygen uptake in the cerebrum than in the other parts of the brain. This confirms the opinion reached previously that the condition in the two parts of the avitaminous brain is different [see Kinnersley and Peters, 1930, 1] and ourselves [1931, 1, Appendix], and that the optic lobes and lower parts are more directly connected with the vitamin B_1 deficiency as such.

Normal brains and washed tissues.

The behaviour of normal brain tissue and of washed tissues to vitamin concentrates does not influence the particular facts under discussion in this paper, though it might have some bearing upon their interpretation. The few experiments, which we have made up to the present, do not justify us in drawing any definite conclusions.

Discussion.

Analysis of the behaviour of the minced optic lobes and rest of the pigeon's brain may seem a devious way to search for the nature of vitamin B_1 deficiency, and a test for vitamin B_1. We have been led to it by failures to demonstrate the action of vitamin concentrates upon more obvious tissues with lactic acid

systems, following the work upon the lower parts of the brain [Kinnersley and Peters, 1930, 1]. It may be hoped that these experiments will lead to some easier test for vitamin B_1, which may be specific enough to settle some of the present confusion, inevitable as long as we are unable to assess the precise influence of other factors of the complex upon vitamin B_1 tests. It is possible, though we think unlikely in view of what has been said earlier, that these reactivations are not due to the vitamin. The proof that a catalytic effect is involved decides between the three possibilities discussed in our earlier paper. It is not clear of course what oxidase system is involved, but it is hoped that experiments at present in progress will decide this point.

Accepting the view provisionally that the vitamin B_1 is itself concerned, it may be inferred that it takes part as such in tissue metabolism without the intermediary action of an internal secretion; its well-known presence in tissues is therefore not an accident of storage.

So far as we are aware, this appears to be the first experimental support for a direct relation between a local biochemical lesion and an abnormal condition of the central nervous system. The argument can be briefly stated. Injection of torulin into the cranium very rapidly relieves opisthotonus symptoms; addition of the same *in vitro* to the optic lobes/rest of the brain restores in part the lowered power of oxygen uptake present in avitaminosis. With the same part of the brain is associated especially the increase in lactic acid found by Kinnersley and Peters [1930, 1]; vitamin rapidly induces an increased rate of disappearance of lactic acid in the tissues of the avitaminous animal [Fisher, 1931]. Realising that there is still much that is not clear, the lesion seems evidently to affect the tissue metabolism of carbohydrate, a view which has been often mooted. Perhaps for the first time, we obtain here experimental grounds for belief in the great importance of the antineuritic vitamin B_1 as such in the normal functioning of the central nervous system.

Conclusions.

1. The oxygen uptake of finely minced pigeon's brain tissue (glucose-phosphate solution) is increased on an average some 40 % by performing the estimations in oxygen instead of air, in the case of cerebrum and the mixed optic lobes and parts below from the normal brain. With the avitaminous (B_1) brain, less or even no increase takes place with the mixed optic lobes and lower parts.

2. In the presence of oxygen the lowered oxygen uptakes previously observed in the brain in avitaminosis B_1 are more marked.

3. Partial restoration of oxygen uptake *in vitro* is observed upon addition of vitamin B_1 concentrates to the mixed optic lobes and lower parts of the avitaminous pigeon.

4. This action of the concentrates follows the vitamin B_1 and is inactivated by treatment with alkali as is vitamin B_1. The action is catalytic.

5. The effects are not always obtained with the cerebral tissue, which behaves differently.

6. Vitamin B_1 is directly concerned with the oxidative metabolism of the lower parts of the pigeon's brain.

We are indebted to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants towards this work. We are also grateful to Mr H. W. Kinnersley for the concentrates used, to Mr Meiklejohn for help in some of the experiments, and to Mr Yates of the Statistical Department, Harpenden, for aid in the analysis of the figures.

REFERENCES.

Abderhalden and Vlassopoulos (1931). *Pflüger's Arch.* **226**, 808.
Carter, Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832.
Cramer (1930). Personal communication.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Fisher (1931). *Biochem. J.* **25**, 1410.
Gavrilescu and Peters (1931, 1). *Biochem. J.* **25**, 1397.
—— —— (1931, 2). *J. Physiol., Proc. Physiol. Soc.* (In press.)
Kinnersley and Peters (1928). *Biochem. J.* **22**, 419.
—— —— (1930, 1). *Biochem. J.* **24**, 711.
—— —— (1930, 2). *Biochem. J.* **24**, 1856.

CCXXXII. THE ASSIMILATION OF ALUMINIUM BY THE HUMAN SYSTEM.

By SAMUEL JUDD LEWIS.

From 329 High Holborn, London, W.C. 1.

(*Received September 22nd, 1931.*)

The scientific work relating to the assimilation of aluminium by the animal system has for the most part failed to give satisfaction, even when by weight of evidence it was fairly conclusive. The chief reasons for this are that the quantities of aluminium absorbed are very small, frequently much less than one part in a million parts of blood or tissue and rarely appreciably more, while the purest reagents obtainable normally and employed in making the search can very rarely if ever be obtained quite free from the element in question. The weight of aluminium present in the necessarily large mass of reagents is often greater than that in the material to be extracted.

Attempts to simplify this problem by resort to the spectrograph are presented in the following observations.

The first experiments were directed to discovering how much, if any, aluminium is present in normal blood obtained first from the slaughter-house and then from man, and to finding and estimating suitably small quantities of aluminium mixed well into the blood before drying down.

The experimental procedure ultimately adopted was to ash a few grams of blood in a new silica crucible after it had been prepared by treatment with hydrochloric acid followed with hot concentrated sulphuric acid. The blood was gently ignited until free from carbon; then 10 mg. of the ash, representing approximately 1 g. of blood, were transferred to the lower electrode of an arc-lamp and the spectrum observed in the usual way by means of a large Hilger quartz spectrograph.

Under the conditions of experiment the two principal aluminium lines 3961·540 and 3944·032 Å. were well in evidence, although feeble, when the proportion of added aluminium was one part in a million parts of blood. The lines were still definitely discernible when the concentration was only one-fifth of that named, and in the later experiments with one in ten millions they could be recognised with certainty.

The absence of extraneous aluminium was assured by (*a*) cleaning the crucible as above described, (*b*) using no reagent, and (*c*) taking a comparison spectrogram of the electrodes alone immediately before placing thereon the ash, to serve as a blank. There is one necessary condition which may at first

sight appear to contradict these tenets, namely, the moistening of the ash with a few milligrams of dilute acid, but as the electrodes were similarly moistened, the blank (*c*) was complete; no aluminium was or could be discovered in the 3 or 4 mg. of acid used, even if it did contain the amount of aluminium said to be present in ordinarily pure reagent acid.

Under the conditions of experiment, sheep's blood was usually found to contain about 1–2 parts per million aluminium; human blood was almost invariably free, that is, it contained less than one part in five or ten millions, although occasionally a very small quantity was indicated.

A series of trials with blood charged with various known quantities of aluminium, round about one part per million, showed a well-graduated series of intensities corresponding with the quantities of element added and gave full assurance that the quantitative indications were satisfactory within the limits shown in the following Tables.

In order to ascertain whether aluminium is taken up by the blood from food two men were dieted for 24 hours or more with food supposed to be free from aluminium; then each of them partook of a meal containing a definite quantity of aluminium. Just before the meal a sample of blood was taken; 3 hours after the meal a second sample, a third $4\frac{1}{2}$ hours after, and a fourth sample 48 hours after. Each sample of blood was then carefully ashed and submitted to spectrographic examination.

The results were that no aluminium could be detected in the first normal samples of blood, nor indeed in those taken 3 hours after the meal. In the case of one subject no aluminium was found in either of the two succeeding samples, except a minute trace after 48 hours. With the other subject traces were discovered after $4\frac{1}{2}$ hours and rather more after 48 hours, but the quantity was very small, probably not more than one part of aluminium (Al) in five and three million parts of blood respectively.

Taking several specimens of blood from the same subject was very trying; and when it was found that normal human blood contains usually no aluminium, or only a minimal quantity, it was decided to take advantage of blood from patients undergoing operations, feeding them with aluminium-containing food before or after the operation according to the nature of the specimens required. This procedure eliminated the drawing of more than occasional special specimens of blood. Several patients awaiting operation had included in their daily diet for a convenient number of days one meal of scones containing about 94 mg. of aluminium, the rest of the diet being normal. This course had an important advantage in the patient remaining normal in temperament and digestive function during the ingestion of the aluminium. Blood was taken some time before commencing the feeding and 24 hours after the last meal.

Assuming that there are about 6 kg. of blood in the human body, the proportion of aluminium in the blood, supposing the whole of the aluminium passed into the blood-stream, would be about 15 parts per million, but this is

of course impossible of realisation, and all considered, not more than a tenth of this amount is likely to appear in the blood.

The observations on the analysis of blood were of three types: (*a*) normal blood; (*b*) normal blood into which known quantities of aluminium had been mixed before drying down; (*c*) blood from subjects who had been fed on aluminium-containing scones.

Preliminary tests, made during the development of the method, are collated in Table I. In the earlier ones, the limit of sensitivity was about 1 in 3,000,000, but in the later ones, the limit was 1 in more than 10,000,000. "W." signifies a particular hospital.

Table I.

Subject	Aluminium—parts per million		
	In normal blood	Added	Found
W. 1	6 times 0 3 times say 0·1 or 0·2	—	*v. infra*
W. 2	0	—	—
W. 3	0	1	1·0, 0·6, 1·4, 1·0
W. 4	0, 0	1	1·4, 1·0
W. 5	0, 0	3	*v. infra*
W. 6	0	3	*v. infra*
W. 7	0	—	*v. infra*
W. 8	0	3	3·0, 3·0
W. 9	—	3	3·0, 3·0, 3·0, 3·0
W. 10	0	1	1·0
W. 11	0	1	0·6

W. 5 and 6. The method for the aluminised ash was modified, with a view to concentrating the Al, by washing away the soluble portion. The results were unduly low, 1·7 and 1·4 respectively, whence it is assumed that the aluminium is partly in a soluble form, possibly as alkaline aluminate, but this was not studied.

W. 7. 8 g. of this specimen were ashed at full temperature in a closed apparatus, in which the vapours evolved were aspirated through water contained in absorption tubes, to ascertain whether any iron was thus vaporised in the form of ferric chloride. No iron was detectable in the distillate by the usual reagents. From this it was inferred that since iron is not volatilised under the conditions, it is probable that aluminium would not be volatilised.

Blood from two sheep was examined. Numerous preliminary experiments showed that the blood from sheep No. 1 contained about 3 parts of Al per million; that from sheep No. 2 about 1 part.

Results of the principal series of experiments on human subjects are collated in Tables II and III. They demonstrate that aluminium is absorbed by the blood of a subject who eats food containing aluminium, and that the amount absorbed does not increase appreciably by continuing the feeding, unless one case ("L") is to be regarded as representative of a certain class of subjects.

"N" shows an exceptionally high figure before feeding, and "G" excep-

tionally low after feeding. The extraordinarily high results with "L" cannot be explained; the surgeon believed the specimen to be satisfactory. In the cases of "α" and "β" the numbers in brackets signify the number of hours after the single meal was taken. The fifth exps. with α and β were by a special method, and the indications were so slight as to be doubtful.

The increase in the aluminium content of the blood is in some degree a measure of the quantity of aluminium which has passed into the blood-stream.

Table II.

Subject	Sex and age	Aluminium found before feeding		
		1st exp.	2nd exp.	Adopted
α	M.	0	0	0
		0 (3rd exp.)	0 (4th exp.)	
β	M.	0	0	—
		0 (3rd exp.)	0 (4th exp.)	0
A	M. 56	0·3	0	0·15
B	M.	0	—	0
E	—	0	—	0
G	M. 25	0	—	0
L	F.	0·1	—	0·1
M	F.	0	—	0
N	M. 25	0·2	0·3	0·25

Table III.

Subject	No. of daily meals	Aluminium found after feeding, parts per million			
		1st exp.	2nd exp.	3rd exp.	Adopted
α	1 (3)	0	0	0	0
		0 (4th exp.)	0·1 (5th exp.)		
	1 ($4\frac{1}{2}$)	0·3	0·4	0·2	0·3
	1 (48)	0·5	0·1	—	0·3
β	1 (3)	0	0	0	0
		0 (4th exp.)	0·1 (5th exp.)		
	1 ($4\frac{1}{2}$)	0·2	0·1	0·1	0·1
	1 (48)	0·3	0·7	—	0·5
A	1	1·2	1·1	1·3	1·2
B	2	0·4	0·2	0·2	0·3
E	5	0·2	0·2	0·5	0·3
G	7	0	0·1	0	0·03
L	12	5	4	—	4·5 (*v. infra*)
M	13	1·2	0·7	1·3	1·1
N	14	0·2	0·2	0·6	0·3

To what extent it is a measure is uncertain at present, because there are no data showing how quickly the aluminium is eliminated again. The observations with the subjects α and β signify that the passage is not very rapid, since it was not until 4 or 5 hours after feeding that the element was first discoverable, nor is the elimination very rapid, seeing that the aluminium lines were stronger at the end of 48 hours than after 4 or 5 hours. The results with subject A are in harmony with the foregoing; at the end of 24 hours the spectrograph revealed quite a substantial proportion, an increase of at least one part per

million. A notable feature of the results is that the quantity of aluminium after feeding is the same however many days the feeding has been continued.

Aluminium absorbed by the milk.

Experiments were made on two women, three cows and one goat. One of the cows and the goat were used twice, at times sufficiently far apart to allow of their being considered separate subjects, making in effect eight in all.

The order of experimenting was in general similar to that followed with blood. The aluminium was administered in combination with the food, as for the blood inquiry. In the case of cows turned out for the summer they were drenched with $\frac{1}{2}$ oz. of alum.

All the results (Table IV) show clearly that aluminium passes fairly rapidly

Table IV. *Aluminium in milk.*

Subject	Before or after feeding	Dose, as alum	Interval (hours)	Al found; parts per million	Remarks
Cows:					
"Gay"	Before	—	—	(*a*) 0 (*b*) 0	After drenching with alum
,,	,,	—	—	(*c*) 0·3 (*d*) 0	(in June)
,,	After	(1¼ ozs.)	9	(*a*) 0 (*b*) 0·4	,,
,,	,,	—	—	(*c*) 0·3 (*d*) 1·0	,,
"Brindle"	Before	—	—	(*a*) 0·3 (*b*) 0	,,
,,	,,	—	—	(*c*) 0 (*d*) 0·1	,,
,,	After	(1¼ ozs.)	9	(*a*) 0·5 (*b*) 0·7	,,
,,	,,	—	—	(*c*) 0·3 (*d*) 0·1	,,
"Gay"	Before	—	—	(*a*) 0·2 (*b*) 0	—
,,	After	1½ ozs.	9	(*a*) 0·9 (*b*) 0·4	—
"Cowslip"	Before	—	—	(*a*) 0 (*b*) 0·1	—
,,	After	1½ ozs.	9	(*a*) 0·8 (*b*) 0·5	—
Goat A	Before	—	—	(*a*) 0 (*b*) 0	—
,,	After	⅜ oz.	5½	(*a*) 0 (*b*) 0	—
Goat B	Before	—	—	(*a*) 0 (*b*) 0	—
,,	After	½ oz.	14	(*a*) 0·3 (*b*) 0·2	—
Human A	Before (?)	—	—	(*a*) 0·1 (*b*) 0·1	As judged by the results
,,	After (?)	28 grains	3½	(*a*) 0 (*b*) 0·02	these two samples have
Human B	Before	—	—	(*a*) 0 (*b*) 0	been transposed; possibly
,,	After	28 grains	2	(*a*) 0·05 (*b*) 0·15	in the nursing home

into the milk. The procedure was to take the first sample of milk just before feeding with aluminised food, and the second sample from the next milking several hours later.

The intervals in the case of the human specimens were very short, and it is quite likely that this accounts for the negative results with subject A, especially with so small a dose, but it may be these two samples were transposed somehow, possibly in the nursing home.

It is interesting that the goat showed no absorption of aluminium the first time, and very little the second, although the dose was fairly large for the size of the animal.

The quantities found compare quite well among themselves if one omits two or three anomalous figures for the cows which were drenched, but with

these first specimens the work was largely of the nature of trial, and might quite fairly be omitted from the report, but they are included so that the whole of the information gleaned may be available.

There are various conditions to be observed in a closer study of the subject; for example, to discover the best interval between the time of feeding and the following milking, and whether the aluminium appears in the earlier or the later stages of the secretion. Also we have no knowledge as to how far the magnitude of the dose or its repetition affects the results. This is a matter of great physiological interest.

Summary.

1. The chief feature of this inquiry is the application of the modern quartz spectrograph, in the absence of all reagents, to the estimation of aluminium in tissues.

2. It has been demonstrated that normal blood usually contains no aluminium, and when it does contain any, the proportion is very small.

3. A considerable quantity of aluminium is found in the blood of the same person after feeding with even one meal of aluminium-containing food.

4. Similar observations apply to the milk.

CCXXXIII. NOTE ON THE ACTIVATION OF OXIDO-REDUCTIONS BY CO-ZYMASE.

By HANS v. EULER and RAGNAR NILSSON.

From the Biochemical Institute of the University of Stockholm.

(*Received September 23rd, 1931.*)

The question of the existence of a co-reductase has frequently been discussed. The differences of opinion upon careful examination have proved often to originate from the fact that this problem has been understood in quite a different way by different authors. In a paper which recently appeared, Harden and Macfarlane [1931] have returned to this question and summarise their results as follows.

"The addition of lactate, succinate or methylglyoxal restored the power of reducing methylene blue to yeast preparations washed to a point at which no fermentation of hexosediphosphate occurred. From these results it is concluded that a co-enzyme is probably not required for the action of the reductase in yeast preparations."

We found previously [Euler, Nilsson and Runehjelm, 1927] in full agreement with Harden and Macfarlane, that the reduction of methylene blue, when performed in presence of lactate and succinate, takes place even in the absence of co-zymase.

On the other hand, co-zymase is required as an activator of the carbohydrate redoxase [Euler and Nilsson, 1926, 1, 2]. It was previously pointed out by Nilsson [1930], that a direct comparison between oxido-reductions in yeast and in muscle, in which different substrates take part as hydrogen donators is justified only in special cases. "Dazu kommt noch, dass die Specifität der bei verschiedenen Donatoren wirksamen Redoxasen nicht genügend erkannt wurde, so dass z. B. die Verbrennung von Milchsäure oder von Bernsteinsäure mit der Verbrennung von Kohlenhydratabbauprodukten in der Hefe und im Muskel direkt verglichen wurde, ohne dass man sich darüber klar machte, dass bei diesen verschiedenen Reaktionen vielleicht in sämmtlichen Fällen ganz verschiedene Enzyme oder Enzymsysteme wirksam sind."

Harden and Macfarlane state, that on the addition of co-zymase and hexosediphosphate to washed yeast fermentation occurs which may give rise to easily oxidisable reaction products, which in their turn cause a reduction of the methylene blue.

In such a case this reduction would be of a secondary character and it would consequently not correspond to the directly activated reaction in which the co-zymase acted as a co-reductase.

The possibility of explaining the chemical mechanism of the reduction of methylene blue in yeast in the manner recently suggested by Harden and Macfarlane has by no means been overlooked by us; it is noted in one of our earliest papers on this subject [Euler and Nilsson, 1926, 1].

However, our more recent results speak more and more in favour of the opinion that the reaction in which the co-zymase directly takes part is of an oxido-reductive character. As this view has already been discussed in a previous publication we only wish to emphasise here that reductions, which are activated by the co-zymase, take place even when fermentation or glycolysis is completely stopped by specific poisons. The product of the oxidation reaction which appears in this case has the composition of a monophosphoglyceric acid [Nilsson, 1930].

This conclusion is supported by the following facts.

Table 73 (p. 92) and Table 81 (p. 98) of Nilsson's paper show the reduction of acetaldehyde in presence and in absence of co-zymase after addition of NaF.

Table 74 (p. 93) shows that NaF completely inhibits all fermentation. From Tables 74 and 81 it will be seen that the reduction of acetaldehyde does not occur without co-zymase and that this reduction is not inhibited by NaF, when added in quantities which completely inhibit fermentation. A comparison with Table 72 (p. 92) shows that on the contrary the reduction of acetaldehyde is activated by such concentrations of NaF.

Since it became clear to us that the transformation of acetaldehyde in dried yeast consists in the reduction of the aldehyde, we used acetaldehyde instead of methylene blue in our oxido-reduction experiments, because it is possible to follow with this substance the course of the reduction from time to time.

We quote here, however, one of our earlier experiments with methylene blue, which shows that, in agreement with our reduction experiments with acetaldehyde, reduction of methylene blue by washed yeast + purified co-zymase and hexosediphosphate occurs in presence of fluoride.

Apozymase + phosphate + hexosediphosphate + methylene blue		Reduction time
+ Purified co-zymase	without NaF	1·5 minutes
	with NaF	1·75 ,,
Without co-zymase		More than 3 hours

The difference between the reduction times 1·5 and 1·75 lies within the limits of the errors of observation.

Concerning our opinion on the reactions which occur in this case and in the case of a normal fermentation we refer to the publication of Nilsson quoted above [Nilsson, 1930] and to a summarising paper by Euler and Nilsson [1930].

In this connection the question whether or not co-zymase acts as an activator of aldehydemutase is of special interest.

The fact that acetaldehyde, added to a suspension of dried yeast, disappears, was at first interpreted by us as a simple dismutation of the aldehyde and because co-zymase was found to be indispensable for the reaction the opinion was expressed that co-zymase has the character of a co-mutase [Euler and Myrbäck, 1927], which assumption was also in agreement with the result that co-zymase is identical with the co-enzyme of the reductase.

This question entered a new stage when it became apparent by the experiments of Nilsson that the acetaldehyde disappears in the presence of dried yeast, not by a simple dismutation but by a reduction very probably of the same type as the reduction of methylene blue.

This new fact caused a modification of our former theoretical views on this subject as is seen from Nilsson's paper [1930].

Thus the rôle of the co-zymase in oxido-reductions in the presence of yeast is cleared up to a certain degree; such is not the case with regard to the transformation of acetaldehyde in the liver, studied in detail by Parnas [1910]. By estimations of alcohol and acid Parnas has shown that it is here probably a question of a real aldehyde dismutation. But as all Parnas's experiments were carried out with mashed liver, or with raw liver juice, thus, with an enzymic material containing rather large amounts of carbohydrates, it is possible that a dismutation of mixed character also takes place, which may have influenced his results to some extent.

In this Institute the question has been again treated by Euler and Brunius [1928]. The most important results then obtained seem to be that purified and dialysed enzyme solutions, themselves inactive, were able to catalyse the aldehyde transformation after the addition of purified co-zymase.

Continuing this investigation we wished to compare the aldehyde transformation which is catalysed by the liver enzyme with the mixed dismutation as it takes place in suspensions of dried yeast.

These experiments also seem to support the opinion that in the liver a real acetaldehyde mutase occurs, which only acts in the presence of co-zymase. The fact that the transformation of acetaldehyde is activated by hexosediphosphate indicates, however, that an oxido-reduction may also occur analogous to that in fermentation by dried yeast, where hexosemonophosphate is proved to take part in a mixed dismutation with acetaldehyde [Nilsson, 1930].

Our results now obtained will be seen from the following tables.

1. *Action of phosphate in presence and in absence of hexosediphosphate.*

Enzyme solution: dialysed liver juice. Co-zymase: 140 Co/cc. ACo = 40,000.
Concentration of the acetaldehyde: 1·9 %.
Phosphate buffer: 0·67 M; $p_H = 6{\cdot}4$.
Concentration of the hexosediphosphate: solution of sodium candiolin, approximately 6 %.
Volume of the reaction mixture: 10 cc. In this volume 1 cc. acetaldehyde solution + 3 cc. enzyme solution + 2 cc. solution of co-zymase.

Acetaldehyde transformed in 2 cc. of the reacting mixture (mg.).

	No hexosediphosphate		1·5 cc. hexosediphosphate	
Mins.	No phosphate	2·5 cc. phosphate	No phosphate	2·5 cc. phosphate
30	0·89	0·83	0·83	1·42
60	1·44	1·48	1·61	2·27
90	1·94	2·17	2·08	2·76

2. *Action of co-zymase in presence and in absence of hexosediphosphate.*

All reagents as in the table above.

Volume of the reaction mixture: 10 cc., containing 1 cc. solution of acetaldehyde + 3 cc. solution of dialysed liver juice + 2·5 cc. phosphate buffer.

Acetaldehyde transformed in 2 cc. of the reacting mixture (mg.).

	No hexosediphosphate		1·5 cc. hexosediphosphate	
Mins.	No co-zymase	2 cc. co-zymase	No co-zymase	2 cc. co-zymase
30	0·10	0·85	0·25	1·40
60	0·12	1·55	0·39	2·25
90	0·23	2·21	0·76	3·03

SUMMARY.

1. For aldehyde transformation, when catalysed by dialysed liver juice and purified co-zymase, phosphate is not required. There is thus a difference from the mixed dismutation, as it occurs in solutions fermented by dried yeast.

2. The transformation of acetaldehyde in dialysed liver juice is increased by the addition of hexosediphosphate or of phosphate.

3. Both in the presence and in the absence of hexosediphosphate co-zymase is indispensable for the transformation of acetaldehyde by liver juice.

We are indebted to Mr Bengt Andersson for valuable help in the experiments recently repeated.

REFERENCES.

Euler and Brunius (1928). *Z. physiol. Chem.* **175**, 52.
—— and Myrbäck (1927). *Z. physiol. Chem.* **165**, 28.
—— and Nilsson (1926, 1). *Z. physiol. Chem.* **162**, 72.
—— —— (1926, 2). *Z. physiol. Chem.* **162**, 264.
—— —— (1930). *Skand. Arch. Physiol.* **59**, 201.
—— Nilsson and Runehjelm (1927). *Z. physiol. Chem.* **169**, 123.
Harden and Macfarlane (1931). *Biochem. J.* **25**, 818.
Nilsson (1930). *Svenska Vet. Akad. Arkiv Kemi* 10 A, nr. 7.
Parnas (1910). *Biochem. Z.* **28**, 274.

CCXXXIV. "RESTING" BACTERIA.

By BRIAN RATCLIFFE SANDIFORD
and WALTER REGINALD WOOLDRIDGE.

From the Divisions of Bacteriology and of Biochemistry, the London School of Hygiene and Tropical Medicine.

(*Received October 13th, 1931.*)

During recent years much work has been reported upon the existence and properties of various bacterial oxidising enzymes. For most of these researches thick suspensions of well-washed bacterial cells in physiological saline or Ringer's solution have been used. These bacterial preparations, when used under conditions such that growth cannot occur [Quastel and Whetham, 1924], have been called "resting" bacteria, a term suggested by analogy with that of "resting" muscle, a similar preparation of well-washed muscle tissue. Although the term "resting" bacteria appears to be justified in the biochemical sense it has been a somewhat unfortunate choice for the bacteriologist if it be taken to imply that "resting" bacteria are normal viable cells which are only unable to proliferate because they are used under conditions unsuitable for their growth; *e.g.* in the absence of necessary food materials or at unfavourable temperatures. It has seemed to him unjustifiable to describe bacterial suspensions as "resting" when no effort was made in the early work to demonstrate any connection between the different stages of the experiments and the total and viable counts of the organisms present. At that time the main concern was to simplify the complexity of bacterial processes as much as possible so that certain of their systems could be more rigorously examined, and hence the actual relationship between the enzymic mechanisms and the life or death of the cells present was not emphasised. In 1927 Quastel and Wooldridge [1927, 1, 2] showed that bacteria previously submitted to various treatments, *e.g.* toluene, chloroform, permanganate *etc.*, so that they would no longer proliferate when inoculated on to agar slopes, nevertheless exhibited little variation in the enzymic activity of many of the mechanisms studied. In these experiments no actual counts were made. After discussing at some length the question of whether the activations of the cell are due to the latter acting as an organised whole, the authors conclude that "we have indeed no evidence to support a contention that the reactions of the organism are dependent upon its survival as a living unit...." Later Cook and Stephenson 1928], who were examining the behaviour of bacterial suspensions in Ringer's solution in a Barcroft differential manometer, *i.e.* in the presence of oxygen,

carried out a series of bacterial counts on their preparations both before and after treatment with ultra-violet light and compared these counts with the oxygen uptake. They conclude "that the oxidation phenomena studied are the work of dead equally with living organisms."

The bulk of the work with "resting" bacteria has been effected under anaerobic conditions, usually at 45°, and it was thought advisable to make total and viable counts of organisms used under these conditions, not only at the beginning and end of the experiments, but at various intervals over a period of several hours. The importance of these Thunberg tube-methylene blue experiments to the bacteriologist was emphasised as early as 1925 [Quastel and Wooldridge, 1925; Quastel and Stephenson, 1925] by the correlation of the results obtained from a study of the enzymic powers of various organisms with their ability to grow upon synthetic media. Since then the term "resting" bacteria has invaded bacteriological literature to such an extent that it has become more than ever necessary to demonstrate the true nature of these suspensions from the angle of the strict bacteriologist. Accordingly the work described in this paper was undertaken.

Experimental.

An organism is said to activate a substrate, examined by the anaerobic methylene blue technique, when the methylene blue is decolorised within a period of 2 hrs., whilst a control experiment with the organism in the absence of the substrate does not reduce the methylene blue at all. Frequently the reduction time with an activated substrate is only a few minutes, but as occasionally experiments have been carried out, and the results reported, over a period of 22 hrs. it was decided to examine the organism over this long period. Accordingly series of Thunberg tube experiments have been put up with *B. coli* suspensions in the presence of various substrates and these have been incubated anaerobically at 45° for different periods of time ranging up to 22 hrs. At the end of the incubation periods total and viable counts of the organisms present in the suspensions have been made. In parallel with these experiments other series of tubes have been incubated so that the effect of the incubation period upon the enzymic activity of the organism could be ascertained. In this way it has been possible to obtain curves showing the variation of total and viable counts with the period of incubation and also to compare these variations with any changes discovered in the enzymic activity.

In the preparation of the organism an 18-hr. broth culture of *B. coli* was inoculated into Roux bottles containing caseinogen digest broth. After 36 hrs. incubation at 37° the organism was centrifuged free of broth, washed twice by centrifugation with sterile saline, made up to a suitable suspension with sterile saline, aerated for half-an-hour, and the organism then stored at 0° until required for use. All experiments were made with an organism that had not been prepared for more than 5 days. For actual use

the suspension was adjusted, by comparison with turbidity controls of *B. coli*, so that its final dilution in the Thunberg tube would contain between 500 and 1000 million cells per cc.

For the Thunberg tube experiments each tube contained 2 cc. of sterile phosphate buffer, p_H 7·4, 1 cc. of sterile substrate, *M*/20 glucose, lactate, formate or succinate—all adjusted to p_H 7·2 and 1 cc. of the *B. coli* suspension. Some of the tubes were incubated with 1 cc. of methylene blue (1/5000 solution in water) whilst to the others 1 cc. of sterile physiological saline was added instead. The organism was added last, the tube closed, immediately evacuated, the side-arm filled with sterile water and placed in the water-bath at 45°. The first of each series of experiments was only left in the bath for 30 secs. when it was removed, cooled under running water, the water removed from the side-arm and the tube carefully opened so that no grease was allowed to descend into the fluid. Total (*i.e.* living and dead cells) and viable counts were begun on this suspension almost immediately. Viable counts were made similarly after incubation for the following times: 20 mins., 40 mins., 1 hr., 2 hrs., 3 hrs., 4 hrs., 5 hrs., 6 hrs. and 22 hrs. Total counts were estimated after 1-hr., 6-hr. and 22-hr. incubation periods. At times various tubes of a series were examined for purity by inoculating nutrient media and incubating both at 37° and at 45° and sometimes at 53° in order to ascertain whether any thermophilic or other contaminants were present. In all these experiments the suspensions were found to consist solely of *B. coli*.

For assessing the total number of organisms present the technique followed was that described by Wilson [1922]. In this the Helber chamber is used and the unstained organisms are counted against a dark background.

The viable counts were made by the agar roll-tube method discussed in the same paper.

All dilutions were effected by the use of dropping pipettes [Donald, 1915]. The margin of error of the results given in the present paper may be slightly greater than usual owing to the fact that at times the dropping pipettes became contaminated with grease, which would affect somewhat the size of the drops.

The experiments for examining the effect of the anaerobic incubation at 45° upon the activity of the enzymes were carried out in Thunberg tubes just as for the bacterial counts. At the end of the required incubation the tube was cooled, the water in the side-arm withdrawn and the stopper carefully opened. The stopper and neck of the tube were cleaned with a dry rag and the former re-greased. If the incubation had taken place in the absence of methylene blue this was now added, the tube closed and re-evacuated and the time required for the reduction of the dye on replacing in the bath at 45° noted. If the first incubation had taken place in the presence of methylene blue, then before the second evacuation the contents of the tube were well shaken to oxidise the dye, and just before immersion in the bath the proportion of oxidised dye present was ascertained by comparison with a series

of controls. Any necessary correction was then made in the second reduction time before comparing this with that previously obtained.

Results representative of the many obtained are set forth in the following tables or shown graphically in the figures.

Discussion of results.

Viable counts. The curves of Fig. 1 show that the number of viable organisms present in bacterial suspensions incubated anaerobically at 45° in the presence of various substrates, but in the absence of methylene blue, decreases with increase in the period of incubation.

If, in addition to the substrate, methylene blue be added, there is a fairly rapid diminution in the number of viable organisms within the first hour, but this is followed by an increase in numbers in all instances except with

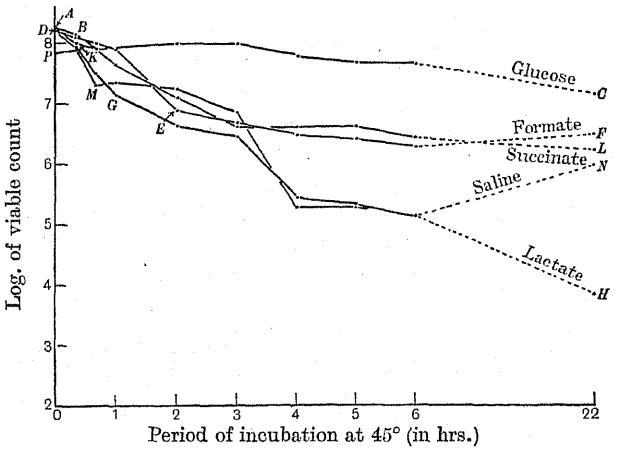


Fig. 1. Showing the relationship between the number of viable organisms and the previous period of incubation at 45° in the presence of the named substrate but in the absence of methylene blue. Glucose = curve *ABC*. Formate = curve *DEF*. Lactate = curve *DGH*. Succinate = curve *AKL*. Saline = curve *PMN*. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

the saline series (see Fig. 2). This increase tends to balance the previous decrease but subsequently the viable count diminishes again. With the saline-methylene blue series no growth at all was obtained from the dilutions planted from the 2-hr. to 6-hr. tubes, but this was probably the result of diluting the suspensions too much, for with the 22-hr. tube, where the suspension was inoculated undiluted, some growth, although less thick than that obtained in the earlier tubes, was noted. It is therefore evident that the increase in the number of viable organisms observed with the other series is absent from that with saline and methylene blue.

It may be possible to explain these results from a consideration of the number of viable organisms involved. There is evidence to suggest [see Bail, 1929] that many organisms will only grow upon media, provided that the

initial concentration of viable organisms is below a certain critical value. This fact might explain the results obtained with the lactate and formate series, for if the initial concentration of viable organisms was already at the critical value in the experiments without the dye then no growth would be expected but in the methylene blue tubes the initial viable count is much less, possibly below this critical "saturation" value, and growth might take place. It is more difficult to explain the glucose results in this way as with these the initial concentrations of viable organisms differ little.

An alternative biochemical explanation may be suggested, depending upon the presence of methylene blue in those tubes where an increase in number of viable organisms is noticed. Methylene blue, in addition to its known inhibitory effect on many enzymes, will also act as a hydrogen acceptor and so will facilitate the oxidation of such hydrogen donators as glucose, lactate and formate in the presence of the organism. These oxidation changes may

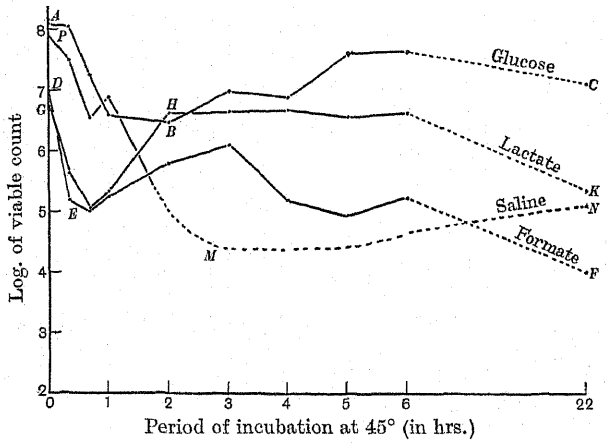


Fig. 2. Showing the relationship between the number of viable organisms and the previous period of incubation at 45°, in the presence of a substrate and of methylene blue. Glucose = curve *ABC*. Formate = curve *DEF*. Lactate = curve *GHK*. Saline = curve *PMN*. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

result in the formation of some substance essential to or definitely stimulatory of growth and would, in addition, yield a supply of energy and, as a result of one or both of these factors, growth might ensue. This growth would be facilitated by the presence of some nitrogenous materials probably made available from the dead cells. Growth will only occur when in addition to the hydrogen acceptor, methylene blue, a hydrogen donator is present and hence no increase in the viable count would be expected in the saline-methylene blue series of experiments—an expectation borne out by experiment. But even in the presence of a hydrogen donator growth will not continue for long under the conditions of these experiments for two reasons; firstly the methylene blue will soon be entirely reduced to the leuco-compound and, although this might be re-oxidised by protein products, the action would continue for but a short time; secondly the inhibitory action of the dye would become increasingly

important. Hence after a short period of growth the number of viable organisms would again decrease. This early cessation of growth might, of course, be due to other factors, such as the lack of adequate nitrogenous material. The above explanation, however, is tentative and needs further work for corroboration. A further fact that should be noticed is that the delay in growth corresponds to the ordinary lag phase of bacterial growth on nutrient media and so the changes noticed may be due to the same causes as are responsible for this lag phase. What relation the phenomena described in the above explanation of the results obtained may have to the lag phase, if any, still remains to be determined.

An examination of the figures given in Table I shows that the total number of cells remains practically stationary throughout the period of incubation. At the beginning the ratio of viable organisms to total cells varies between 1 : 2 and 1 : 7 in the different experiments, except with the lactate-methylene blue series, but at the end of the period of incubation this ratio is always greatly altered—varying between 1 : 200 and 1 : 6,000,000. It follows therefore that if the enzymic activity of these suspensions were examined in parallel with the growth series, then the enzymic activity should either be little changed, if it is independent of whether the cell is alive or dead, or it should fall off greatly and become immeasurable by our technique if the activity is due solely to the presence of living organisms. This holds only if the velocity of the enzyme reaction as measured by the Thunberg technique is proportional to the concentration of the enzyme present. That this is so is shown by the curves given in Fig. 3. The results given in Table II show that for practical purposes the enzymic activity remains constant, the greatest change recorded being a sixfold increase in the reduction time. These results therefore conclusively demonstrate that the enzymic activity is not solely dependent upon the living cell but appears to be a property both of cells that are unable to grow, even given suitable conditions, and of cells that are still alive and only unable to grow during the experiment because of the conditions of this experiment. The implication that preparations described as "resting" bacteria consist mainly of cells which are in a state of suspended animation as regards growth is incorrect, although the claim that the synthetic reactions associated with growth are largely inhibited by their use under specified conditions remains valid. The term "resting" bacteria therefore should signify a suspension of washed bacterial cells, some alive and some dead, used under conditions such that proliferation, that is increase in the number of living cells, is inhibited during the usual period of observation. Then the reactions studied will generally be those associated with both the living and the dead cells.

As the cardinal point in this definition is that multiplication of the bacteria is inhibited during the use of "resting" bacteria it is obviously essential, if the term be applied at all generally to the use of suspensions of washed bacterial cells, that control experiments on bacterial counts be conducted in parallel with the biochemical reactions being studied. Under conditions such

Table I. *Showing the effect of varying the substrate upon the total and viable counts of organisms present in suspensions of* B. coli *incubated anaerobically at 45°. Except for the series marked with an asterisk, the results given (no. per cc.) with one substrate were obtained with the same bacterial suspension.*

	Substrate		Count	0 min.	20 mins.	40 mins.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	22 hrs.
1.	Saline		Total	363,000,000	—	—	323,000,000	—	—	—	—	327,000,000	330,000,000
2.	,,	+M.B.	Total	333,000,000	—	—	330,000,000	—	—	—	—	357,000,000	367,000,000
3.	,,	+M.B.	Viable	73,720,000	30,510,000	3,390,000	8,052,000	0	0	0	0	0	133,900
4.	,,	*	Viable	66,900,000	83,030,000	42,450,000	23,030,000	16,940,000	7,340,000	191,220	205,700	125,700	983,700
5.	Lactate		Total	1,000,000,000	—	—	1,220,000,000	—	—	—	—	1,212,000,000	894,000,000
6.	,,		Viable	171,000,000	123,700,000	62,700,000	44,910,000	11,870,000	5,932,000	308,600	343,000	291,000	25,710
7.	,,	+M.B.	Total	1,018,000,000	—	—	1,034,000,000	—	—	—	—	876,000,000	1,040,000,000
8.	,,	+M.B.	Viable	5,453,000	471,500	120,000	222,900	4,286,000	4,629,000	4,887,000	3,517,000	4,286,000	214,500
9.	,,	*	Viable	210,000,000	90,000,000	33,000,000	14,600,000	4,661,000	3,000,000	271,500	225,000	132,000	7,310
10.	Formate		Total	616,000,000	—	—	654,000,000	—	—	—	—	570,000,000	566,000,000
11.	,,		Viable	194,800,000	46,610,000	67,790,000	10,590,000	9,745,000	4,661,000	34,300	154,300	56,010	8,570
12.	,,	+M.B.	Total	694,000,000	—	—	724,000,000	—	—	—	—	674,000,000	450,000,000
13.	,,	+M.B.	Viable	9,322,000	1,611,000	124,300	185,700	638,300	1,277,000	165,000	85,270	178,400	9,840
14.	,,	*	Viable	152,400,000	119,900,000	93,240,000	74,730,000	8,664,000	5,484,000	3,368,000	2,887,000	1,987,000	3,273,000
15.	Glucose		Total	832,000,000	—	—	1,002,000,000	—	—	—	—	762,000,000	858,000,000
16.	,,		Viable	169,400,000	135,500,000	77,900,000	266,400,000[1]	103,400,000	96,580,000	64,390,000	50,860,000	45,750,000	13,560,000
17.	,,	+M.B.	Total	830,000,000	—	—	878,000,000	—	—	—	—	747,000,000	628,000,000
18.	,,	+M.B.	Viable	118,600.000	110,100,000	18,880,000	4,237,000	2,966,000	10,590,000	7,628,000	38,140,000	43,220,000	118,870,000
19.	,,	*	Viable	13,920,000	10,490,000	10,670,000	10,250,000	7,509,000	7,750,000	6,991,000	2,030,000	140,700	—
20.	Succinate		Viable	165,900,000	111,100,000	84,330,000	43,190,000	12,850,000	4,230,000	4,268,000	3,210,000	2,762,000	1,663,000

[1] The suspension in this tube was badly affected with grease.

as those used by Quastel and Wooldridge [1927, 1, 2; 1928] in which the organisms are initially exposed to high concentrations of inhibitors such as toluene, $KMnO_4$ *etc.*, this precaution is not so necessary. But the fact that growth has been observed to take place under conditions thought to be inhibitory of any increase in numbers, as described in the present paper, makes such controls generally advisable. It is true that this increase in numbers

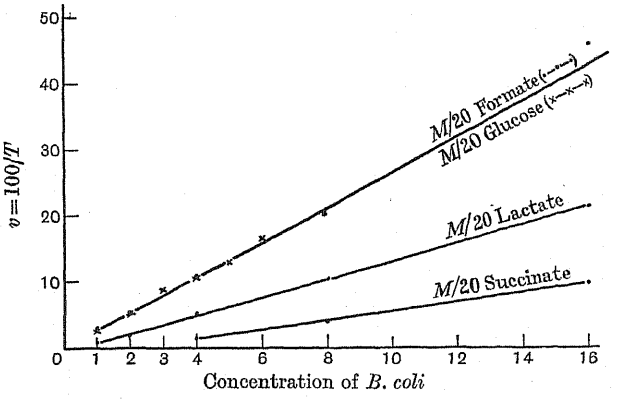


Fig. 3. Showing the relationship between the concentration of "resting" *B. coli* and the velocity of reduction of methylene blue ($v=100/T$, where T=time of reduction in mins.) in the presence of various substrates. Concentration 16 of *B. coli* = approx. 5×10^8 cells per cc.

Table II. *Showing the effect of an initial anaerobic incubation of variable duration at 45° and* p_H *7·4 upon the times (in mins.) taken by "resting"* B. coli *to reduce* M/*25,000 methylene blue solution, the preliminary incubation taking place in the presence or the absence of the dye, as noted at the head of each column.*

Period of first incubation at 45°	Lactate	Lactate +M.B.	Formate	Formate +M.B.	Glucose	Glucose +M.B.	Succinate	Succinate +M.B.
0 min.	10	8	8	9	13	12	8	8
5 mins.	10	12	12	17	15	15	6	8·5
10 ,,	8·5	9	7	7	13	14·3	7	8
15 ,,	12	7	7	8	11	19	8	8
20 ,,	9	11	8	8·5	11	21	10	8
30 ,,	12	10	11	7	14	13	10	11
45 ,,	13	10	8	6·5	11·3	12	14	12
1 hr.	11	13	6·5	8	11	12	14	15
2 hrs.	8	14	7	9	10	14·3	20	21
3 ,,	14	10·5	6·5	8	12·5	11·3	18	20
4 ,,	11	16	6·5	11	12·5	10·7	18	22
5 ,,	10	16	7	6·5	12	9·5	14	20
6 ,,	11	18	7	8	10	12·7	19	21
22 ,,	20	49	9	18	12·3	17·7	51	70

was small and transient and that it only occurred after the usual period of observation in these enzyme studies. (In the great majority of dehydrogenase experiments reported in the previous work upon these bacterial enzymes the period of observation did not exceed 30 mins. and so the conclusions of, and deductions drawn from, this work are in no way invalidated.) Nevertheless, owing to the recent extension of the use of so-called "resting" bacteria,

especially in America, it seems advisable to discontinue the use of this term before absolute confusion results. The only justification for the use of the term is if bacterial growth definitely does not take place, and as this is generally doubtful and rarely proven it is preferable that future workers along these lines should simply allude to the use of washed bacterial cells and describe the conditions under which they are employed.

Summary.

1. It is shown that preparations described as "resting" bacteria consist both of cells that will proliferate when inoculated upon suitable media and of cells that are unable to grow under such conditions.

2. The total number of cells remains practically constant throughout long periods of anaerobic incubation at 45° in the presence of various substrates with or without methylene blue.

3. In the absence of the dye the number of viable organisms steadily decreases, whereas in the presence of methylene blue the viable count first shows a fall followed by a temporary rise, subsequently falling more slowly. An explanation of this phenomenon is suggested.

4. The enzymic activity of these preparations towards the various substrates used is shown to remain practically constant throughout the period of incubation.

5. The enzymic activities examined in this work are not solely dependent upon the presence of living cells; both viable and dead cells are active.

6. As the number of living cells may sometimes increase under conditions thought to be inhibitory of growth it is essential that control experiments assessing the number of viable organisms be conducted whenever the term "resting" bacteria is used.

7. It is preferable to discontinue the use of this term entirely and to refer simply to washed bacterial cells used under certain specified conditions.

REFERENCES.

Bail (1929). *Z. Immunität.* **60**, 1.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Donald (1915). *Lancet*, ii, 1243.
Quastel and Stephenson (1925). *Biochem. J.* **19**, 660.
—— and Whetham (1924). *Biochem. J.* **18**, 519.
—— and Wooldridge (1925). *Biochem. J.* **19**, 652.
—— —— (1927, 1). *Biochem. J.* **21**, 148.
—— —— (1927, 2). *Biochem. J.* **21**, 1224.
—— —— (1928). *Biochem. J.* **22**, 689.
Wilson (1922). *J. Bact.* **7**, 405.

CCXXXV. THE NITROGEN METABOLISM OF THE LUPIN SEEDLING.

By PHYLLIS McKIE.

From the Department of Biochemistry, Westfield College, London.

(*Received October 21st, 1931.*)

The following investigation was undertaken for two reasons; to follow the nitrogen metabolism of the young plant through successive stages of growth and thus to arrive at some estimate of the part played by different nitrogenous metabolites, and to provide a preliminary survey for the much more complicated problem—at what stage and by what means is the pyrrole ring synthesised in the plant?

The first stages of germination of a seed containing reserve protein involve the breakdown of that protein. It becomes a matter of considerable interest therefore to examine the various products of this breakdown and to gain some knowledge of the nitrogen compounds which are produced by the plant from its reserves, prior to their use in synthesis. It is apparent, as will be shown, that protein synthesis begins after about the 18th day of germination, when the seed reserves have been (for the lupin) largely depleted. The main seat of metabolic activity of such synthesis is probably in the leaves. In a young seedling, in which the leaves form a small proportion of the bulk of the plant, it is misleading to confine analysis solely to these parts, and up to the 82nd day of growth the whole seedling has been taken for analysis. The material from each sowing (about 12 plants) was mixed, discarding any obvious abnormalities. In this way minor differences, inseparable from the growth of individual plants, were eliminated. In the lupin, asparagine represents about one-half of the protein decomposition products in the seed, and the nitrogen metabolism of the lupin seedling is therefore peculiar in this respect, but asparagine is probably only one of several labile intermediate substances of protein breakdown, and the subsequent course of protein re-synthesis may give some qualitative idea of this process in other seedlings.

Experimental.

Lupin seeds (*Lupin luteus*), were grown in large pots containing a granular soil and sand mixture in a cool greenhouse throughout the summer and early autumn of 1930. Twelve pots, each producing ultimately about a dozen plants, were used for each sowing. Regulation of the temperature of the greenhouse ensured approximately the same conditions throughout the experiment. In

one lot only were conditions adverse; Lot 1, a 31 day period lasting from October 25 to November 29, which coincided with an exceptionally cold spell of weather. Artificial heat above the average cool temperature was not favourable, and growth was stunted accordingly.

Preparation and analysis of material.

The plants were released from the soil by turning out the pot and gently disengaging the root and root-hairs as much as possible without breaking. Ten minutes' immersion in water and very gentle cleaning with the fingers were sufficient to free the seedlings entirely from soil, They were quickly air-dried on sheets of blotting-paper, and measured for total length, number of leaves and lateral roots.

After 1 minute's immersion in boiling water to arrest growth and enzyme action, the seedlings were dried as completely as possible on blotting-paper and transferred to spirit. This enables analyses to be undertaken at leisure, and avoids the disadvantage of high temperature drying of the partially wet material; it also affords a means of isolating the alcohol-insoluble asparagine which appears in quantity after a few days' growth. A deposit of asparagine appears at the bottom of the jar, and in the subsequent stages—rapid air-drying at 50°—the rest of the asparagine crystallises out on the dry material, and can readily be detached. The asparagine-free material is ground to powder in an end-runner mill and dried at 100° to constant weight. Analyses for total nitrogen (Kjeldahl) only were carried out on the dry material.

Extraction of material. The dried and weighed material was intimately ground in an end-runner mill with 100 cc. of ammonia-free water for 20 minutes and filtered through fine muslin. The process was repeated twice more, the last residue being pressed as dry as possible.

Residue. This was dried at 100° to constant weight and analysed for total nitrogen.

Filtrate. The combined filtrates were made up to 500 cc. with ammonia-free water, made antiseptic with a little toluene and analysed.

Total nitrogen. Of the dry material weights averaging 0·02 g., or of the extract 10 cc. evaporated to dryness in the Kjeldahl flask, were taken. The modified Kjeldahl method used by Robinson [1929], was found satisfactory. A clear liquid was always obtained after 20 minutes' boiling, and boiling was continued for a further 10 minutes. Distillations were carried out in the usual apparatus with the addition of 30 cc. of ammonia-free water, 5 cc. of 25 % sodium sulphide, 5 cc. of N copper sulphate, and 25 cc. of alcohol, aerated by acid-washed air during the 20 minutes' boiling, the ammonia being received in $N/10$ H_2SO_4. Controls had established the accuracy of this procedure.

Protein. 10 cc. of extract were diluted to 100 cc. with absolute alcohol, added slowly with constant shaking. After standing for 36 hours the precipitated protein was collected in a Jena glass crucible and dried in a desiccator to constant weight. Nitrogen was calculated in this protein as 12·8 %.

Proteose. The method of Wasteneys and Borsook [1917] was found most suitable. 20 cc. of extract were employed.

Nitrate. This was determined on 50 cc. of extract by reduction with Devarda's alloy as recommended by Chibnall [1922].

Amino-acids. The Sørensen method of estimation of amino-acid content was finally adopted, 10 cc. being used for each titration.

Ammonia. Free ammonia, which is usually small in amount, is best estimated by the Folin aeration method, using solid anhydrous sodium carbonate. 30 cc. of extract *plus* 2 g. of anhydrous sodium carbonate were aerated in the Folin apparatus for 1 hour at 15°, the ammonia being received in 25 cc. of $N/100$ H_2SO_4.

Amides. The amide content was taken as represented by that amount of ammonia *minus* the free ammonia, which is yielded by the Folin method after 5 hours' boiling with 4 % HCl, by the method of Sachsse [1873]. 5 cc. of extract and 20 cc. of acid were taken for each estimation.

Asparagine. A considerable proportion of the asparagine, when present, crystallises out from the material in spirit, and, after the removal of the seedlings, can be filtered off through a Jena glass crucible, dried and weighed. The remainder crystallises on the surface of the seedlings during the air-drying and can be detached with a small brush, collected and weighed.

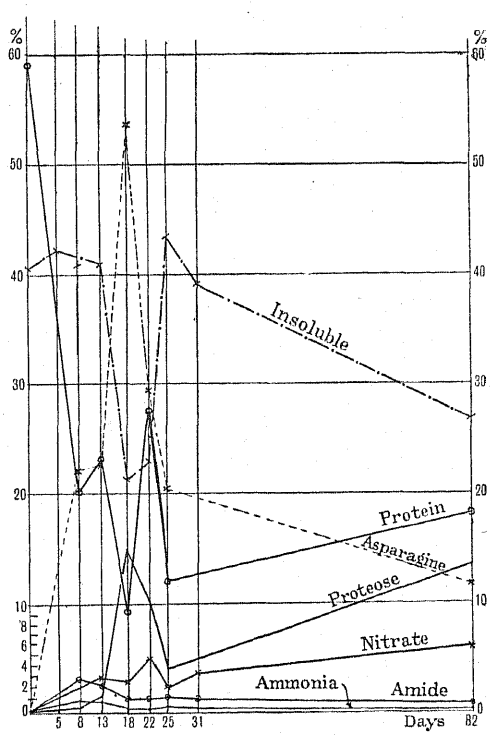


Fig. 1.

Examination of the curves, Fig. 1, shows that over a period of 81 days' growth from the seed, protein, insoluble nitrogen compounds, and asparagine represent by far the greater part of the total nitrogen.

In the seed, soluble protein and nitrogenous material insoluble after the three extractions with water account for the whole of the nitrogen.

After 5 days' growth, soluble protein has decreased sharply, and this is the more marked at 8 days, when there has been a fall of protein nitrogen from 59·2 % (in the seed) to 20·2 %. After a slight fluctuation, the level falls still further, until at the 18th day it is only 9·8 %, the minimum value (Table I).

Table I. *Variation in protein content.*

	Age (days)	Protein dry weight g.	Protein as % of total solid	Protein-nitrogen as % of total solid	Protein-nitrogen as % of total nitrogen
X.	0	13·4	29·6	3·8	59·2
B.	5	—	—	—	—
A.	8	0·395	12·95	1·65	20·02
F.	13	0·420	16·21	2·07	23·4
C.	18	0·410	14·68	1·88	9·81
G.	22	0·740	35·34	4·52	27·8
E.	25	0·320	13·69	1·75	12·01
D.	31	0·735	18·5	2·24	15·03
H.	82	0·665	22·37	2·34	18·5

(N.B. Protein nitrogen is calculated as 12·8 %.)

Following closely this decrease in protein there is a sharp decrease in insoluble nitrogenous material, which reaches its minimum on the 22nd day at 14·9 % (original seed value = 41·09 %) (Table II).

Table II. *Variation in content of insoluble nitrogen compounds.*

Age (days)	Nitrogen as % of dry residue	Residue-nitrogen as % of total dry weight	Residue-nitrogen as % of total nitrogen
0	8·8	2·64	41·09
5	—	4·94	43·96
8	8·8	3·98	61·3
13	7·07	3·61	41·5
18	6·7	3·97	20·7
22	5·3	2·42	14·9
25	8·54	6·34	43·55
31	8·22	5·8	38·9
82	5·29	3·33	26·3

Concomitantly with this fall asparagine increases rapidly during the early days of growth. It is absent from the ungerminated seed, but after 18 days' growth the asparagine represents 53·7 % of the total nitrogen. This is its maximum value, and during subsequent growth it diminishes rapidly in the next 12 days, corresponding to a synthesis of protein, and thereafter diminishes slowly. Asparagine appears, therefore, to represent the chief metabolic product in the first stages of growth and to represent, with the protein, the greater part of the nitrogenous material of the seedling (Table III).

Table III. *Variation in asparagine content.*

	Age (days)	Total solid dry wt. g.	Asparagine g.	Asparagine-nitrogen g.	Asparagine-N as % of dry wt.	Asparagine-N as % of total N
X.	0	—	0	0	0	0
B.	5	—	—	—	—	—
A.	8	3·0487	0·2521	0·0535	1·75	21·23
F.	13	2·5924	0·2377	0·0503	1·94	22·3
C.	18	2·7928	1·3292	0·2818	10·29	53·7
G.	22	2·0945	0·4724	0·1002	4·78	29·4
E.	25	2·3367	0·3146	0·0667	2·99	20·5
D.	31	3·9735	0·4087	0·0867	2·18	14·6
H.	82	2·9729	0·189	0·04	1·35	10·6

Proteoses are seen to be an immediate decomposition product of the protein and insoluble nitrogenous material, the curve for proteose nitrogen rising from zero in the seed to a maximum at 14·2 % after 18 days' growth. While protein is being rapidly synthesised during the next 7 days, proteoses diminish, but in the subsequent slower growth of the young plant, proteoses increase gradually with a corresponding increase of protein, keeping as would be expected, at a lower level throughout (Table IV).

Table IV. *Variation in proteose content.*

Age (days)	Total nitrogen of extract g.	Nitrogen other than protein or proteose g.	Nitrogen of protein *plus* proteose g.	Nitrogen of protein g.	Nitrogen of proteose g.	Proteose-nitrogen as % of total solid	Proteose-nitrogen as % of total nitrogen
5	0·198	0·127	0·071	0·050	0·021	0·07	0·85
8	0·175	0·084	0·091	0·053	0·038	0·15	1·72
13	0·253	0·125	0·128	0·052	0·076	2·72	14·2
18	0·241	0·112	0·129	0·095	0·034	1·68	10·3
22	0·273	0·220	0·059	0·046	0·013	0·55	3·78
25	—	—	—	—	—	—	—
31	0·335	0·218	0·117	0·069	0·048	1·66	13·13
82	—	—	—	—	—	—	—

The behaviour of the labile metabolic products shows an interesting parallel. Always low and never representing in total more than 9 % of the total nitrogen, and averaging ammonia and amino-acids, 0·2 %, amides and nitrates 3 %, they appear in the seedling after 5 days' growth, and are at their maximum during the period of rapid change, 8 to 18 days, corresponding to the very rapid fall of protein and the equally rapid rise of asparagine. With the exception of nitrates they maintain a fairly constant low level throughout the subsequent slower periods of growth. Nitrates tend to increase slightly as the seedling acquires more leaves and leaf metabolism thereby increases (Tables V, VI, VII and VIII).

The apparently insignificant part played in the seedling by amides, amino-acids, nitrates and ammonia, as suggested by the curves, is probably however misleading, for at no time is it to be expected that these substances, as much the elements of synthesis as of degradation, would accumulate. Thus, while the breakdown of protein to amino-acids, amides and ammonia may be rapid

Table V. *Variation in amide content.*

	Age (days)	Total solid dry wt. g.	N as amide *plus* ammonia g.	N as ammonia g.	N as amide g.	Amide-N as % of total solid	Amide-N as % of total nitrogen
X.	0	—	0	0	0	0	0
A.	8	3·0487	0·0105	0·0026	0·0079	0·259	3·15
F.	13	2·5924	0·0084	0·0021	0·0063	0·243	2·76
C.	18	2·7928	0·0091	0·0014	0·0077	0·276	1·41
G.	22	2·0945	0·0063	0·0014	0·0049	0·234	1·44
E.	25	2·3367	0·007	0·0013	0·0057	0·244	1·65
D.	31	3·9735	0·0091	0·0012	0·0079	0·199	1·34
H.	82	2·9729	0·0056	0·0014	0·0042	0·141	1·11

Table VI. *Variation in ammonia content.*

	Age (days)	Total solid dry wt. g.	Ammonia g.	Ammonia nitrogen g.	Ammonia-N as % of total solid	Ammonia-N as % of total nitrogen
X.	0	—	0	0	0	0
A.	8	3·0487	—	0·0026	0·084	1·02
B.	5	2·3628	—	0·00059	0·0257	0·26
F.	13	2·5924	—	0·0021	0·081	0·93
C.	18	2·7928	—	0·0014	0·050	0·22
G.	22	2·0945	—	0·0014	0·067	0·41
E.	25	2·3367	—	0·0013	0·055	0·38
D.	31	3·9735	—	0·0011	0·029	0·19
H.	82	2·9729	—	0·0014	0·047	0·73

Table VII. *Variation in nitrate content.*

	Age (days)	Total solid dry wt. g.	Nitrate-nitrogen g.	Nitrate-nitrogen as % of dry wt.	Nitrate-nitrogen as % of total nitrogen
X.	0	—	0	0	0
B.	5	—	—	—	—
A.	8	—	—	—	—
F.	13	2·5924	0·0071	0·2746	3·15
C.	18	2·7928	0·0156	0·56	2·92
G.	22	2·0945	0·0171	0·8155	5·01
E.	25	2·3367	0·0071	0·3044	2·09
D.	31	3·9735	0·0199	0·5013	3·36
H.	82	2·9729	0·0227	0·7656	6·01

Table VIII. *Variation in amino content.*

	Age (days)	Total solid dry wt. g.	Amino-nitrogen g.	Amino-nitrogen as % of dry wt.	Amino-nitrogen as % of total nitrogen
X.	0	0	0	0	0
B.	5	—	—	—	—
A.	8	3·0487	0·0023	0·076	0·92
F.	13	2·5924	0·0022	0·083	0·954
C.	18	2·7928	0·0016	0·056	0·29
G.	22	2·0945	0·0008	0·040	0·24
E.	25	2·3367	0·0010	0·044	0·30
D.	31	3·9735	0·00103	0·026	0·17
H.	82	2·9729	0·0005	0·016	0·13

and extensive, the removal of these compounds in an actively growing plant would be equally rapid. Thus the curve representing their presence at any one stage would be expected to show a low and fairly constant percentage.

Table IX. *Distribution of nitrogen as percentage of total nitrogen.*

	Age (days)	Total nitrogen %	N as NH_3 %	N as nitrate	N as amide	N as protein	N as proteose	N as amino-compounds	N as asparagine	Insol. N	Total
X.	0	6·42	0	0	0	59·2	0	0	0	41·1	100·3
B.	5	9·89	0·26	—	—	—	—	—	—	43·9	—
A.	8	8·24	1·02	—	3·15	20·2	0·85	0·92	21·2	—	—
F.	13	8·7	0·93	3·1	2·76	23·4	1·72	0·95	22·3	41·5	96·6
C.	18	19·16	0·22	2·9	1·41	9·81	14·2	0·29	53·7	20·7	103·2
G.	22	16·29	0·41	5·03	1·41	27·8	10·3	0·25	29·4	24·9	99·4
E.	25	14·56	0·38	2·06	1·65	12·0	3·7	0·30	20·5	43·5	84·2
D.	31	14·49	0·19	3·36	1·34	15·0	—	0·17	14·6	38·9	—
H.	82	12·64	0·37	6·01	1·11	18·5	13·1	0·13	10·6	26·3	—

The rôle of asparagine in the metabolism of the leguminous seed has been investigated by several workers. Schulze [1880, 1911; Schulze and Winterstein, 1910] have noticed its formation in considerable quantity concomitantly with the breakdown of protein on germination. They suggest that its removal corresponds to a re-synthesis of protein, but this has not, so far, received quantitative treatment. Chibnall [1924], in his examination of the mature leaf of the runner bean, finds the same phenomenon and suggests that asparagine is a translocation product whereby nitrogen is conveyed from regions of protein catabolism to those of re-synthesis. The close relationship, shown by the curves, Fig. 1, between the fall of asparagine and proteose and protein re-synthesis in the lupin seedling is striking confirmation of this.

The seed, during the rapid degradation of reserve protein, concentrates its labile nitrogen in the form of the soluble, and hence mobile, compound asparagine. When the peak of catabolic activity is passed, and the young plant has energy free for the building up of its own material, asparagine is quickly removed by further chemical change, and synthesis of proteose and protein begins, passing presumably through the stages of amide, nitrate and ammonium salt.

Summary.

1. Insoluble nitrogenous material, protein and asparagine, represent the bulk of the total nitrogen of the lupin seedling in the seed and in the early stages of growth.

2. A close relationship between decrease of insoluble and protein nitrogen and increase of asparagine is observed.

3. Protein synthesis begins after 16 days' growth and is accompanied by a fall in asparagine content and a rise in proteose content.

4. Ammonia, amides, amino-acids and nitrates are absent from the ungerminated seed. They appear immediately growth begins, rise to a low maximum in a few days and then maintain a constant lower level.

REFERENCES.

Chibnall (1922). *Biochem. J.* **16**, 344.
—— (1924). *Biochem. J.* **18**, 395.
Robinson (1929). *Biochem. J.* **23**, 1099.
Sachsse (1873). *J. prakt. Chem.* **6**, 118.
Schulze (1880). *Landes. Jahrb.* **9**, 689.
—— (1911). *Z. physiol. Chem.* **71**, 31.
—— and Winterstein (1910). *Z. physiol. Chem.* **65**, 431.
Wasteneys and Borsook (1917). *J. Biol. Chem.* **29**, 25.

CCXXXVI. THE BASIC AMINO-ACIDS OF WOOL.

By A. MALCOLM STEWART and CLAUDE RIMINGTON.

From the Wool Industries Research Association, Headingley, Leeds.

(*Received August 3rd, 1931.*)

Only two determinations have been made of the basic amino-acids present in wool, Marston [1928] using the indirect Van Slyke method found a constant proportion of basic nitrogen in several samples of wool analysed. His figures for histidine, arginine and lysine were 6·9, 10·2 and 2·8 % respectively. Vickery and Block [1930], in a careful series of determinations by direct isolation from a sample of wool, found 7·8 % of arginine, 2·3 % of lysine and only 0·66 % of histidine. In their paper they comment upon the fact that other keratins such as human hair have been found to contain about 0·5 % of histidine [Vickery and Leavenworth, 1929], whereas Marston's figures are over ten times as great.

Marston's figures were reported with some reservation, owing to the well-known difficulty of obtaining trustworthy results by the Van Slyke method, particularly when the protein under examination is one containing a large proportion of cystine.

With regard to the amount of arginine in wool, Marston mentioned that "the estimation of arginine by silver precipitation and by gravimetric means utilising flavianic acid as a precipitating agent yielded figures which were quite constant and at considerable variance with the figures yielded by the above methods" (Van Slyke determinations).

In order to clear up the discrepancy, it was thought advisable to verify Vickery and Block's results by a repetition of the determinations using the modified procedure described by Calvery [1929] for the quantitative isolation of the basic amino-acids from small quantities of protein (5 g.).

In the present investigations a single sample of wool has been used, successive duplicate determinations by the Calvery method being carried out and also a direct colorimetric determination of histidine by the Hanke and Koessler [1920] procedure.

The figures obtained by both methods were in close agreement, averaging 0·5 %, and thus confirming those of Vickery's analyses of wool and of human hair.

Experimental.

A fleece from a West Australian 2 years old Merino ewe was scoured by warm steeping in soap and water, thoroughly washed with liberal quantities of water in a hydro-extractor and finally run through a small carding machine

which not only removed much foreign material but also tended to mix the sample. After hand-carding it was picked free from any residual vegetable matter *etc.*

From the 250 g. of wool so prepared, 1 g. samples (duplicate) were taken for ash and 0·1 g. portions (triplicate) for total nitrogen determinations.

Moisture determinations were carried out upon 1 g. samples dried to constant weight at 105° in a stream of dry air.

	Moisture %	Ash %	Total nitrogen* %
	10·66	0·62	16·50
	10·63	0·62	16·43
	—	—	16·37
Mean	10·65	0·62	16·43

* Corrected for ash and moisture.

It was found in a preliminary trial that when 5 g. of wool were boiled with 25 cc. of 20 % hydrochloric acid, maximal amino-nitrogen liberation was attained within 12 hours. There was no change up to 24 hours' boiling, the proportion of nitrogen present in the amino-form being, at this stage, 62·5 % as determined by formaldehyde titration and 82·47 % by reaction with nitrous acid ($\frac{1}{2}$ hour in the Van Slyke apparatus).

It is of interest to note that the non-amino-nitrogen calculated on the assumption of 4·4 % of proline, 1·8 % of tryptophan, 7·8 % of arginine, and 0·6 % of histidine works out at 16·23 % in good agreement with the 17·5 % calculated from the Van Slyke figures.

Determination of the bases by means of their silver salts etc.

Hydrolysis was effected by boiling 5 g. wool with 50 cc. of 20 % hydrochloric acid for 18 hours, after which Calvery's procedure was followed. Whenever possible loss of material was guarded against by testing washings of precipitates, crude fractions *etc.*, by means of the appropriate colour reactions—Sakaguchi's test for arginine, the diazo-reaction for histidine and the ninhydrin colour test for amino-acids in solution.

In this way the importance was realised, as emphasised by Calvery, of keeping a strongly acid reaction whilst adding silver oxide and in making distinctly alkaline to phenolphthalein when precipitating the arginine silver salt.

Some inconvenience was caused by the occasional precipitation of silver sulphide arising from the partial decomposition of some cystine in the presence of silver hydroxide. It was removed by filtration.

Of the crude histidine fraction, 50 cc. in all, 25 cc. were taken for total nitrogen, 24 cc. for isolation of the flavianate and 1 cc. diluted to 10 cc., of which 1 cc. aliquots were used for colorimetric histidine determinations by the Hanke and Koessler method.

The Congo red-methyl orange standard was checked against pure histidine hydrochloride and was entirely satisfactory.

As noticed by the other workers, the figures for total nitrogen of the purified fractions were in excess of the quantities of the bases actually isolated. The impurity in the first lysine fraction was particularly large.

Results of determinations.

	Histidine (%)		
Exp. No.	By total nitrogen	By flavianate	By colorimetric determination
1	0·736	0·59	0·54
2	0·733	0·62	0·51

	Arginine (%)	
Exp. No.	By total nitrogen	By flavianate
1	6·64	5·60
2	6·0	5·97

	Lysine (%)	
Exp. No.	By total nitrogen	By picrate
1	7·23	(1·86)*
2	2·24	2·8

* Portion lost.

Histidine by colorimetric determination.

2·5 g. of wool were boiled for 24 hours with 50 cc. of 20 % hydrochloric acid. The hydrolysate was made up to 100 cc. and total nitrogen determined on 2 cc. aliquots. The procedure outlined by Hanke and Koessler was then followed exactly, the final solution of basic phosphotungstate being diluted to 500 cc. Colorimetric determination was carried out upon 1 cc. samples.

Results.

	Histidine
	0·55 %
	0·55 %
Mean	0·55 %

As there was only 0·04 g. of histidine flavianate available for isolation, the values given by the colorimetric method are probably the more reliable.

Discussion.

No claim is made that the proportions of the basic amino-acids present do not vary from wool to wool, since variations as great as 18 % are known to occur in the cystine content of Australian Merino wool [Barritt and King, 1929]. It is clear however that the percentage of histidine is of the order found by Vickery and Block; namely, 0·5–0·6 %.

Since, up to the present, all attempts to discover variability in the amino-acid content of different wools have been confined to cystine alone, the

realisation is important that this variation may well be influenced by variation in any other amino-acid present in the keratin. It would be of interest to carry out a comparative series of determinations of histidine and other easily determined amino-acids in a series of wools of different origin. Constancy of composition is hardly to be expected, nor is it required by the hypothesis put forward by Astbury and Woods [1931] as to the nature of the linkage of amino-acids in wools.

SUMMARY.

1. The basic amino-acids in a fleece of Australian wool have been estimated by Calvery's method. The values for histidine have been verified by Hanke and Koessler's colorimetric method.

2. This wool gave 0·55 % histidine, 6 % arginine and 2·2 % lysine.

3. These figures are in agreement with those given by Vickery and his co-workers for wool and for human hair and support his opinion "that Marston has been led to an erroneous conclusion with respect to the proportion of histidine yielded by wool."

A. M. S. of the University of Western Australia wishes to thank the Council and Director of the Wool Industries Research Association for facilities granted him for working temporarily in their laboratories.

REFERENCES.

Astbury and Woods (1931). *Phil. Trans. Roy. Soc. Lond.* A **230**, 75.
Barritt and King (1929). *J. Text. Inst.* **20**, 151 T.
Calvery (1929). *J. Biol. Chem.* **83**, 631.
Hanke and Koessler (1920). *J. Biol. Chem.* **43**, 527.
Marston (1928). Council Sci. and Ind. Research, Commonwealth of Australia, Bull. 38.
Vickery and Block (1930). *J. Biol. Chem.* **86**, 107.
—— and Leavenworth (1929). *J. Biol. Chem.* **83**, 523.

INDEX

Absorption of water by gelatin (Jordan-Lloyd) 1580; (Pleass) 1943
Accessory factor necessary for growth of *Nematospora gossypii* (Buston and Pramanik) 1656, 1671
Acid-denaturation of haemoglobin (Meldrum) 1498
Acidosis, chlorides and inorganic constituents of serum and cerebrospinal fluid in (Linder and Carmichael) 1090
Adrenaline, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Ågren, G., Wilander, O. and Jorpes, E. Cyclic changes in the glycogen content of the liver and the muscles of rats and mice. Their bearing upon the sensitivity of the animals to insulin, and their influence on the urinary output of nitrogen 777
Ahmad, B. The fate of carotene after absorption in the animal organism 1195
Aitken, H. A. A. A further note on the determination of iodine in blood 446
Alcock, R. S., *see* Cook, R. P.
Alcohols, secondary, paraffins and ketones, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
Algae, marine, occurrence of sugar alcohols (dulcitol) in (Haas and Hill) 1470
Allchorne, E., *see* Bacharach, A. L.
Allen, L. A. Observations on some alcohol-soluble proteins from milk products 1045
Aluminium, assimilation of, by the human system (Lewis) 2162
Aluminium, excretion and absorption of, in the rat (Mackenzie) 287
Aluminium in aluminium protein compounds, a method for determining (Pope) 1949
Amino-acids, basic, of wool (Stewart and Rimington) 2189
Amino-acids, Erlenmeyer synthesis of (Lamb and Robson) 1231
Ammonia, oxidation of, in percolating filters (Jenkins) 147
Ampholytes, effect of addition of salts on p_H of solutions of (Giedroyć and Przyłęcki) 465
Amylase and lactic acid formation in muscle, relation between (Case) 561
Amylase-starch-gelatin system (Freiberger) 705
Amylase-starch-protein systems, action of salts on (Chrempińska) 1555
Anslow, W. K. and Raistrick, H. Studies in the biochemistry of micro-organisms. XIX. 6-Hydroxy-2-methylbenzoic acid, a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx 39
Anthocyanin of *Oxycoccus macrocarpus* Pers. (Grove and Robinson) 1706
Anthocyanins, survey of (Robinson and Robinson) 1687
Antiglyoxalase (Giršavičius) 1807
Antimony trichloride test of liver oils (Gillam and Morton) 1346
Antiscorbutic factor, reprecipitation of (Zilva) 594
Antiscorbutic potency of apples (Bracewell, Kidd, West and Zilva) 138; (Bracewell, Wallace and Zilva) 144
Apple cuticle, wax constituents of (Chibnall, Piper, Pollard, Smith and Williams) 2095
Apple, determination of reducing sugars in, iodimetrically (Archbold and Widdowson) 101
Apples, antiscorbutic potency of (Bracewell, Kidd, West and Zilva) 138; (Bracewell, Wallace and Zilva) 144
Archbold, H. K. and Widdowson, E. M. Iodimetric determination of reducing sugars in the apple 101
Arginine, effect of, on body weight of tumour-bearing mice injected with thyroxine (Gilroy) 414
Arsenate, effect of, on the induction period of zymin (Macfarlane) 822
Ashford, C. A. and Holmes, E. G. Further observations on the oxidation of lactic acid by brain tissue 2028
Aspergillus, production of mannitol from hexoses and pentoses by white species of (Coyne and Raistrick) 1513
Auchinachie, D. W., *see* McGowan, J. P.
Auden, H. A. and Dawson, E. R. The hydrolysis of concentrated sugar solutions by invertase 1909
Autolysis in malignant and normal rabbit tissues (Price) 1491
Avitaminosis B, intoxication by methylglyoxal in (Vogt-Møller) 418

Bacharach, A. L., Allchorne, E. and Hazley, V. The effect of adding vitamin A to a rachitogenic diet 639
B. coli cultures, effect of bacteriophage on oxidation-reduction potentials of (Hewitt) 1641
B. coli, effect of hydrogen ion concentration on oxidations by (Cook and Alcock) 523
B. coli, reduction of nitrates by (Stickland) 1543
B. coli, respiration of (Cook and Haldane) 880
B. coli, respiratory catalysts of, relationship between (Cook, Haldane and Mapson) 534
B. dysenteriae cultures, effect of bacteriophage on oxidation-reduction potentials of (Hewitt) 1447
B. pyocyaneus, action of, on citric acid (Walker, Subramaniam, Stent and Butterworth) 129
Bacteria, liberation of elementary nitrogen by (Barritt) 1965
Bacteria, "resting" (Sandiford and Wooldridge) 2172

Bacteria, types of products formed from glucose by (Birkinshaw, Charles and Clutterbuck) 1522
Bacterial proteases, formation of, in synthetic media (Haines) 1851
Bacteriophage, effect of, on oxidation-reduction potentials of *B. coli communis* cultures (Hewitt) 1641
Bacteriophage, effect of, on oxidation-reduction potentials of *B. dysenteriae* (Shiga) cultures (Hewitt) 1447
Bacteriophage, effect of, on oxidation-reduction potentials of *Staphylococcus* cultures (Hewitt) 2068
Banks, A. and Hilditch, T. P. The glyceride structure of beef tallows 1168
Barger, G. and Weichselbaum, T. E. A new synthesis of methionine 997
Barium, micro-determination of (McLaughlin) 307
Barritt, J. and King, A. T. Note on the sulphur-free nature of medulla in blackface wool 1075
Barritt, J. and Rimington, C. The relation between total sulphur and cystine content of certain samples of rabbit fur 1072
Barritt, N. W. The biological filtration of dilute sucrose solutions 1419
Barritt, N. W. The liberation of elementary nitrogen by bacteria 1965
Barton-Wright, E. C. and Boswell, J. G. The biochemistry of dry-rot in wood. II. An investigation of the products of decay of spruce wood rotted by *Merulius lachrymans* 494
Basic amino-acids of wool (Stewart and Rimington) 2189
Bekker, J. G. and King, A. T. Sulphur distribution in the component structures of wool and porcupine quills 1077
Bell, M. E. The blood-sugar level in vitamin B_1 deficiency 1755
Beriberi, experimental, glyoxalase-co-enzyme ratio in (Vogt-Møller) 418
Bhattacharya, B. and Hilditch, T. P. The body fats of the pig. I. Influence of ingested fat on the component fatty acids 1954
Biological values of proteins of various breads (Kon and Markuze) 1476
"Bios," relation of accessory factor for growth of *Nematospora gossypii* to (Buston and Pramanik) 1671
Birch, T. W. and Guha, B. C. The chemical nature of vitamin B_1 from evidence afforded by its electrical transference 1391
Bird, G. M. and Haas, P. On the nature of the cell wall constituents of *Laminaria* spp. Mannuronic acid 403
Birkinshaw, J. H., Callow, R. K. and Fischmann, C. F. Studies in the biochemistry of micro-organisms. XXII. The isolation and characterisation of ergosterol from *Penicillium puberulum* Bainier, grown on a synthetic medium with glucose as sole source of carbon 1977
Birkinshaw, J. H., Charles, J. H. V. and Clutterbuck, P. W. Studies in the biochemistry of micro-organisms. XXI. Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of bacteria 1522
Bladder, urinary, phosphatase activity of transplants of (Huggins) 728
Blissett, A. H. and Golding, J. On the inadequacy of white wheat flour and fish meal for prolonged growth of pigs and its amelioration with yeast or stout 349
Blood-calcium following intravenous injection of calcium salts (Greville) 1931
Blood corpuscles, red, quantity of free water in (Schiødt) 8
Blood, determination of inorganic sulphate in (Cuthbertson and Tompsett) 1237
Blood, determination of iodine in (Aitken) 446
Blood, ergothioneine content of (Salt) 1712
Blood, factors influencing complement in (Osborn) 2136
Blood-fat, determination of (Himwich, Friedman and Spiers) 1839
Blood glycolysis (Reid and Narayana) 339
Blood-phosphatases (Roche) 1724
Blood-phosphorus in fat absorption (Vahlquist) 1628
Blood-plasma, human, determination of inorganic sulphate in (Cope) 1183
Blood-sugar, determination of, by the Hagedorn-Jensen method (Kramer and Steiner) 161
Blood-sugar in vitamin B_1 deficiency (Bell) 1755
Blow-fly larvae, collagenase from (Hobson) 1458
Boas-Fixsen, M. A. The effect of desiccation upon the nutritive properties of egg-white. II 596
Booth, J., *see* Campbell, W. G.
Boswell, J. G., *see* Barton-Wright, E. C.
Boxwood, proximate analysis and nature of furfuraldehyde-yielding constituents of (Preece) 1304
Boyd, W. J. The nature of the "ether reaction" of urine 812
Bracewell, M. F., Kidd, F., West, C. and Zilva, S. S. The antiscorbutic potency of apples. II 138
Bracewell, M. F., Wallace, T. and Zilva, S. S. The antiscorbutic potency of apples. III 144
Bracewell, M. F. and Zilva, S. S. Vitamin C in the orange and the grape fruit 1081
Brain tissue, oxidation of lactic acid in (Ashford and Holmes) 2028
Briggs, G. E. The significance of the Michaelis constant for saccharase 1801
Bruins, H. R., Overhoff, J. and Wolff, L. K. The molecular weight of vitamin A 430
Buston, H. W. and Pramanik, B. N. The accessory factor necessary for the growth of *Nematospora gossypii*. I. The chemical nature of the accessory factor 1656
Buston, H. W. and Pramanik, B. N. The accessory factor necessary for the growth of *Nematospora gossypii*. II. The relation of the accessory factor to "bios" 1671
Butter-fats, glyceride structure of (Hilditch and Sleightholme) 507
Butterworth, J., *see* Walker, T. K.

Cabbages, lipoid matter extracted from (Collison and Smedley-MacLean) 606
Calcification of the bones of rats on a diet low in ergosterol (Hume and Smith) 292
Calcium, effect of, on rate of diffusion of sugars through surviving intestine (Magee and Sen) 643
Calcium metabolism of guinea-pigs in scurvy (Humphreys and Zilva) 579
Calcium-phosphate intake, influence of, on hypervitaminosis D (Harris and Innes) 367
Calcium salts, blood-calcium following intravenous injection of (Greville) 1931
CALLOW, R. K. The purification of ergosterol 79
CALLOW, R. K. Occurrence of α-dihydroergosterol as an impurity in yeast ergosterol 87
CALLOW, R. K. and FISCHMANN, C. F. The occurrence of vitamin D in lampreys (Petromyzontidae) 1464
CALLOW, R. K. *see also* BIRKINSHAW, J. H. and ROSENHEIM, O.
CAMPBELL, W. G. The chemistry of the white rots of wood. II. The effect on wood substance of *Armillaria mellea* (Vahl.) Fr.; *Polyporus hispidus* (Bull.) Fr., and *Stereum hirsutum* Fr. 2023
CAMPBELL, W. G. and BOOTH, J. The chemical aspect of the drying of timber. 2. The drying of a softwood 756
Cancer tissue, inhibitory effect of monoiodoacetic acid on lactic acid production by (Harrison and Mellanby) 770
CAPPER, N. S., MCKIBBIN, I. M. W. and PRENTICE, J. H. Carotene and vitamin A. The conversion of carotene into vitamin A by fowl 265
Carbohydrate complex of serum-proteins (Rimington) 1062
Carbohydrate content of isolated frog muscle (Kerly) 671
Carbohydrate-free diet, metabolism of animals on (Hynd and Rotter) 1893
Carbohydrate-free diets, sensitivity of animals on, towards insulin (Hynd and Rotter) 457
Carbohydrate, galactose as the dietary (Guha) 1385
Carbohydrate metabolism in birds (Fisher) 1410
Carbohydrates in the sweet potato, chemical changes in, during cooking (Sinoda, Kodera and Oya) 1973
CARMICHAEL, E. A., *see* LINDER, G. C.
Carotene, conversion of, into vitamin A by fowl (Capper, McKibbin and Prentice) 265
Carotene, distribution of, in the body of the rat (Moore) 275
Carotene, fate of, after absorption in the animal organism (Ahmad) 1195
Carotene, vitamin A and (Moore) 2131
CASE, E. M. The relation between amylase and lactic acid formation in muscle 561
CASE, E. M. and COOK, R. P. The occurrence of pyruvic acid and methylglyoxal in muscle metabolism 1319
CASE, E. M., *see also* MCCULLAGH, D. R.
Caseinogen, effect of proteinase of green malt on (Hopkins and Kelly) 256
Caseinogen for vitamin tests (Coward, Key, Dyer and Morgan) 551
Catabolism, increased, distribution of nitrogen and sulphur in urine during (Cuthbertson) 236
Catalase, effect of, on oxidation-reduction potentials of haemolytic streptococcus cultures (Hewitt) 858
Catalase, effect of, on oxidation-reduction potentials of pneumococcus cultures (Hewitt) 169
Catalysis by copper sulphate of oxidation of oleic acid by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Catalysis of oxidation of cysteine and glutathione by iron and copper (Pirie) 1565
Cereals, vitamin B_1 values of (Plimmer, Raymond and Lowndes) 691
Cerebrospinal fluid, chlorides and inorganic constituents of, in nephritis and acidosis (Linder and Carmichael) 1090
CHARLES, J. H. V., *see* BIRKINSHAW, J. H.
CHIBNALL, A. C., PIPER, S. H., POLLARD, A., SMITH, J. A. B. and WILLIAMS, E. F. The wax constituents of the apple cuticle 2095
CHIBNALL, A. C., *see also* PIPER, S. H. and POLLARD, A.
Chick, fat-soluble vitamin requirements of (McFarlane, Graham and Richardson) 358
Cholesterol and phosphatide contents of normal and malignant human tissues (Jowett) 1991
Cholesterol and phosphatides, determination of small amounts of (Jowett and Lawson) 1981
CHREMPIŃSKA, H. Structure and enzyme reactions. X. Action of salts on the systems: amylase-starch-proteins 1555
Chromogenic constituents of fresh and aged liver oils (Heilbron, Gillam and Morton) 1352
Citric acid, action of *B. pyocyaneus* on (Walker, Subramaniam, Stent and Butterworth) 129
Citric acid dehydrogenase of cucumber seeds, temperature coefficients and energy exchanges of (Dann) 177
CLIFFORD, W. M. The loss of glucose from dried peas on soaking 1999
CLUTTERBUCK, P. W., *see* BIRKINSHAW, J. H.
Cod-liver oils, determination of vitamin A in (Coward, Dyer, Morton and Gaddum) 1102
COLE, S. W. A method for the direct determination of urea in urine 1653
Collagenase from blow-fly larvae (Hobson) 1458
COLLIN, G. Some peculiarities in the glyceride structure of laurel fats 95
COLLISON, D. L. and SMEDLEY-MACLEAN, I. The nature of the lipoid matter extracted from green leaves (spinach and cabbage) 606
Colloids, effect of p_H on precipitating and protective actions of mixtures of proteins on (Hopkins, Kermack and Spragg) 829, 1860
Complement in the blood, factors influencing (Osborn) 2136
COOK, R. P. and ALCOCK, R. S. The effect of hydrogen ion concentration on some oxidations by *B. coli communis* 523

Cook, R. P. and Haldane, J. B. S. The respiration of *B. coli communis* 880
Cook, R. P., Haldane, J. B. S. and Mapson, L. W. The relationship between the respiratory catalysts of *B. coli* 534
Cook, R. P., *see also* Case, R. M.
Cooking, chemistry of (Sinoda, Kodera and Oya) 1973
Cope, C. L. Determination of inorganic sulphate in human blood-plasma by microtitration 1183
Copper in animal nutrition (Cunningham) 1267
Coprophagy, effects of, in rats deprived of vitamin B complex (Roscoe) 2056
Coward, K. H., Dyer, F. J., Morton, R. A. and Gaddum, J. H. The determination of vitamin A in cod-liver oils (*a*) biologically, (*b*) chemically, (*c*) physically, with a statistical examination of the results 1102
Coward, K. H., Key, K. M., Dyer, F. J. and Morgan, B. G. E. Caseinogen for vitamin tests 551
Coyne, F. P. and Raistrick, H. Studies in the biochemistry of micro-organisms. XX. On the production of mannitol from hexoses and pentoses by a white species of *Aspergillus* 1513
Co-zymase, activation of oxido-reductions by (v. Euler and Nilsson) 2168
Creed, R. H., *see* Lovern, J. A.
Cunningham, I. J. Some biochemical and physiological aspects of copper in animal nutrition 1267
Cunningham, I. J., *see also* McGowan, J. P.
Cuprous derivatives of some sulphydryl compounds (Pirie) 614
Cuthbertson, D. P. The distribution of nitrogen and sulphur in the urine during conditions of increased catabolism 236
Cuthbertson, D. P. and Tompsett, S. L. A preliminary note on the inorganic sulphate content of the blood with a method for its determination 1237
Cuthbertson, D. P. and Tompsett, S. L. The enzymic synthesis of proteins with special reference to the action of pepsin 2004
Cyanide and other salts, effects of, on fermentation by yeast preparations (Patterson) 1593
Cysteine, catalysis of oxidation of, by iron and copper (Pirie) 1565
Cystine and total sulphur of rabbit fur (Barritt and Rimington) 1072
Cystine, determination of, in proteins (Tompsett) 2014

Damodaran, M. The amino-acids of glutenin 190
Damodaran, M. The dicarboxylic acid-nitrogen of proteins. With a note on the alcohol-soluble protein from ragi (*Eleusine coracana*) 2123
Dann, W. J. The temperature coefficients and energy exchanges of the citric acid dehydrogenase of cucumber seeds 177
Dann, W. J. and Moore, T. The effect of vitamin B deficiency upon the vitamin A reserves of the rat 914
Dawson, E. R., *see* Auden, H. A.
Dehydrogenase, citric acid, of cucumber seeds, temperature coefficients and energy exchanges of (Dann) 177
Dehydrogenases, effect of narcotics on (Sen) 849
Diastase, malt, effect of proteins and protein derivatives on hydrolysis of starch by (Filipowicz) 1874
Dickens, F. and Šimer, F. The metabolism of normal and tumour tissue. III. A method for the measurement of respiratory quotient in serum 973
Dickens, F. and Šimer, F. The metabolism of normal and tumour tissue. IV. The respiratory quotient in bicarbonate-media 985
Diet, rachitogenic, effect of addition of vitamin A to (Bacharach, Allchorne and Hazley) 639
Dietary factors, rôle of, in formation of erythrocytes (Guha and Mapson) 1674
a-Dihydroergosterol, occurrence of, as an impurity in yeast ergosterol (Callow) 87
Dihydroxyacetone (Reeves and Renbom) 412
3 : 4-Dihydroxyphenylalanines, synthesis of the optically active (Harington and Randall) 1028
d-3 : 5-Diiodotyrosine, isolation of, from the thyroid gland by the action of proteolytic enzymes (Harington and Randall) 1032
Dissociation constants of *i*-β-hydroxyglutamic acid (Harington, Randall and Richardson) 1917
Dry-rot in wood, biochemistry of (Barton-Wright and Boswell) 494
Dudley, H. W. Intermediary carbohydrate metabolism. The effect of sodium iodoacetate on glyoxalase 439
Dulcitol, occurrence of, in marine algae (Haas and Hill) 1470
Dunlop, D. M., *see* Stewart, C. P.
Dyer, F. J., *see* Coward, K. H.
Dyestuffs, effect of, on enzymes of oxidation (Quastel and Wheatley) 629
Dyestuffs, effect of, on fumarase (Quastel) 898

Edestin, effect of proteinase of green malt on (Hopkins and Kelly) 256
Egg-albumin, effect of proteinase of green malt on (Hopkins) 245
Egg-white, effect of desiccation upon the nutritive properties of (Boas-Fixsen) 596
Electrodes, "indifferent," behaviour of, in determination of oxidation-reduction potentials in presence of hydrogen (Lepper and Martin) 45
Ellis, R. W. B., *see* Goldblatt, M. W.
Elphick, G. K., *see* Key, K. M.
Enterokinase, effect of heat upon the activating efficiency of (Pace) 1
Enzyme action, the addition compound theory of (Woolf) 342
Enzyme from animal tissues oxidising hexose-diphosphoric acid (Harrison) 1011
Enzyme from blow-fly larvae which digests collagen in alkaline solution (Hobson) 1458
Enzyme, oxidising, from animal tissues (Harrison) 1016
Enzyme reactions, structure and (Freiberger) 705; (Chrempińska) 1555

Enzymes, effect of dyestuffs on (Quastel and Wheatley) 629
Enzymes, trypanocidal action and toxicity to (Quastel) 1121
Enzymic synthesis of proteins with special reference to the action of pepsin (Cuthbertson and Tompsett) 2004
Epidermis, human, permeability of, to ultra-violet irradiation (Lucas) 57
Ergosterol, calcification of the bones of rats on a diet low in (Hume and Smith) 292
Ergosterol, isolation and characterisation of, from *Penicillium puberulum* Bainier, grown on a synthetic medium with glucose as sole source of carbon (Birkinshaw, Callow and Fischmann) 1977
Ergosterol, purification of (Callow) 79
Ergothioneine content of the blood in health and disease (Salt) 1712
Erlenmeyer synthesis of amino-acids (Lamb and Robson) 1231
Erythrocytes, rôle of dietary factors in formation of (Guha and Mapson) 1674
Erythrocytes, sensitisation of, in haemolysis (Sen) 1244
Esterase, liver, inhibitory action of synthetic urethanes on (Stedman and Stedman) 1147
"Ether reaction" of urine, the (Boyd) 812
EULER, H. v. and NILSSON, R. Note on the activation of oxido-reductions by co-zymase 2168
Excretion, nitrogen, effect of insulin on (Goldblatt and Ellis) 221
Exercise, muscular, fat metabolism in (Stewart, Gaddie and Dunlop) 733
EYRE, J. V. Notes on oil development in the seed of a growing plant 1902

Fat absorption, blood-phosphorus in (Vahlquist) 1628
Fat-free diet, relation of, to scaly tail condition in rats (Hume and Smith) 300
Fat metabolism in muscular exercise (Stewart, Gaddie and Dunlop) 733
Fats, body, of the pig. I. Influence of ingested fat (Bhattacharya and Hilditch) 1954
Fats, laurel, peculiarities in the glyceride structure of (Collin) 95
Fats, natural, spectrographic data of, in relation to vitamin A (Gillam, Heilbron, Hilditch and Morton) 30
Fatty acids of cat's kidney (Turner) 49
Fatty acids of natural fats, spectrographic data of, in relation to vitamin A (Gillam, Heilbron, Hilditch and Morton) 30
Fermentation by yeast preparations, effects of cyanide and other salts on (Patterson) 1593
Fermentation, selective, of glucose and fructose (Hopkins) 245
Fibres, outer-coat animal, relation between cystine yield and total sulphur in (Rimington) 71
Fibrin, effect of proteinase of green malt on (Hopkins and Kelly) 256
FILIPOWICZ, B. Influence of proteins and protein derivatives on the enzymic hydrolysis of starch by malt diastase 1874
Filters, percolating, biological oxidation of sucrose and ammonia in (Jenkins) 147
Filtration, biological, of dilute sucrose solutions (Barritt) 1419
FINE, J. On the influence of serum on enzymes, with special reference to its action on trypsin 647
FISCHMANN, C. F., *see* BIRKINSHAW, J. H. and CALLOW, R. K.
Fish meal, inadequacy of, for prolonged growth of pigs (Blissett and Golding) 349
Fish meal, vitamin A and vitamin D content of (McFarlane, Graham and Richardson) 358
FISHER, R. B. Carbohydrate metabolism in birds. III. The effects of rest and exercise upon the lactic acid content of the organs of normal and rice-fed pigeons 1410
Fish-livers, Mittelmann process for treating (Lovern, Creed and Morton) 1341
FREIBERGER, S. Structure and enzyme reactions. IX. The systems amylase-starch-gelatin and urease-urea-gelatin 705
FRIEDMAN, H., *see* HIMWICH, H. E.
Fructose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843
Fruits and vegetables, vitamin B_1 values of (Plimmer, Raymond and Lowndes) 1788
Fruits, distribution of vitamin B complex in (Roscoe) 2050
Fumarase, action of dyestuffs on (Quastel) 898
Fur, rabbit, relation between total sulphur and cystine content of (Barritt and Rimington) 1072

GADDIE, R., *see* STEWART, C. P.
GADDUM, J. H., *see* COWARD, K. H.
Galactose as the dietary carbohydrate (Guha) 1385
Galactose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843
Gallotannin, action of tannase on (Nicholson, Nierenstein, Pool and Price) 752
GAVRILESCU, N. The iodine method for the determination of glutathione 1190
GAVRILESCU, N. and PETERS, R. A. Biochemical lesions in vitamin B deficiency 1397
GAVRILESCU, N. and PETERS, R. A. On the function of torulin. An *in vitro* effect of antineuritic vitamin concentrates 2150
Gelatin, absorption of water by (Jordan-Lloyd) 1580
Gelatin, absorption of water by; combination of gelatin with acids (Pleass) 1943
Gelatin and ovalbumin, electrophoretic velocities of, in their mixtures (Prideaux and Howitt) 391
GIEDROYĆ, G. and PRZYŁĘCKI, S. J. Effect of the addition of salts on the p_H of solutions of ampholytes 465
GIEDROYĆ, W., *see also* PRZYŁĘCKI, S. J.
GILLAM, A. E., HEILBRON, I. M., HILDITCH, T. P. and MORTON, R. A. Spectrographic data of natural fats and their fatty acids in relation to vitamin A 30
GILLAM, A. E. and MORTON, R. A. The antimony trichloride test and the ultra-violet absorption of liver oils and concentrates 1346

GILLAM, A. E., *see also* HEILBRON, I. M.
GILROY, E. The effect of arginine on the body weight of mice injected with thyroxine, and bearing the tumour M 63 414
GIRŠAVIČIUS, J. O. Studies on antiglyoxalase. II. Methods of following glyoxalase action 1807
Glucosaminodimannose, isolation of, from carbohydrate complex of serum-proteins (Rimington) 1062
Glucose dehydrogenase: a new oxidising enzyme from animal tissues (Harrison) 1016
Glucose, determination of renal threshold for (Himsworth) 1128
Glucose, loss of, from dried peas on soaking (Clifford) 1999
Glucose, types of products formed from, by species of bacteria (Birkinshaw, Charles and Clutterbuck) 1522
Glutamic acid peptide from seaweed (Haas and Hill) 1472
Glutathione, catalysis of oxidation of, by iron and copper (Pirie) 1565
Glutathione, iodine method for determination of (Gavrilescu) 1190
Glutathione, metabolism of, in the dog (Hele and Pirie) 1095
Glutenin, amino-acids of (Damodaran) 190
Glyceride structure of beef tallows (Banks and Hilditch) 1168
Glycogen content of liver and muscles of rats and mice, cyclic changes in, in relation to sensitivity to insulin and urinary output of nitrogen (Ågren, Wilander and Jorpes) 777
Glycogen, distribution of, in the rat, effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on (Lawrence and McCance) 570
Glycolysis, blood (Reid and Narayana) 339
Glycosuria, relation of, to glycaemia (Himsworth) 1128
Glyoxalase action, methods of following (Giršavičius) 1807
Glyoxalase-co-enzyme ratio in experimental beriberi (Vogt-Møller) 418
Glyoxalase-co-enzyme ratio in liver tissue in inanition (Vogt-Møller) 1540
Glyoxalase, effect of sodium iodoacetate on (Dudley) 439
Gold, colloidal, effect of hydrogen ion concentration on precipitating and protective actions of proteins on (Hopkins, Kermack and Spragg) 829, 1860
GOLDBLATT, M. W. and ELLIS, R. W. B. The effect of insulin on growth, nitrogen excretion and respiratory metabolism 221
GOLDING, J., *see* BLISSETT, A. H.
GRAHAM, W. R., Jr., *see* MCFARLANE, W. D.
Grape fruit, vitamin C in (Bracewell and Zilva) 1081
Grasses, forage, wax constituents of (Pollard, Chibnall and Piper) 2111
GREVILLE, G. D. Study of the blood-calcium following the intravenous injection of calcium salts 1931
GROLLMAN, A. The vapour pressure depressions of aqueous solutions of phosphate buffer mixtures at 20·3° 166
GROVE, K. E. and ROBINSON, R. An anthocyanin of *Oxycoccus macrocarpus* Pers. 1706
Growth and chemical composition of functioning fowl's ovary (Romanoff) 994
Growth, effect of insulin on (Goldblatt and Ellis) 221
GUHA, B. C. Investigations on the preparation and behaviour of vitamin B_1 concentrates from yeast 931
GUHA, B. C. Investigations on vitamin B_2. I. The sources of vitamin B_2. II. The stability of vitamin B_2. III. The chemistry of vitamin B_2 945
GUHA, B. C. Observations on certain factors necessary for the normal nutrition of the rat 960
GUHA, B. C. The physiological function of vitamin B_1 1367
GUHA, B. C. On galactose as the dietary carbohydrate 1385
GUHA, B. C. and MAPSON, L. W. The rôle of certain dietary factors in the formation of erythrocytes 1674
GUHA, B. C., *see also* BIRCH, T. W.
Gum benzoin, effect of hydrogen ion concentration on precipitating and protective actions of proteins on (Hopkins, Kermack and Spragg) 829, 1860

HAAS, P. and HILL, T. G. The occurrence of sugar alcohols in marine algae. Dulcitol 1470
HAAS, P. and HILL, T. G. A preliminary note on the nitrogen metabolism of seaweeds. Glutamic acid peptide 1472
HAAS, P., *see also* BIRD, G. M.
Haemoglobin, acid-denaturation of (Meldrum) 1498
Haemolysis, sensitisation of erythrocytes in (Sen) 1244
Hagedorn-Jensen-Hanes method for determination of reducing sugars, modification of (Hulme and Narain) 1051
HAINES, R. B. The formation of bacterial proteases, especially in synthetic media 1851
HALDANE, J. B. S., *see* COOK, R. P.
HARDEN, A. and MACFARLANE, M. G. Note on the reactivation of reductase in washed yeast preparations 818
HARDING, V. J. and SELBY, D. L. Fermentable sugar in normal urine 1815
HARINGTON, C. R. and RANDALL, S. S. Synthesis of the *d*- and *l*-3 : 4-dihydroxyphenylalanines 1028
HARINGTON, C. R. and RANDALL, S. S. The isolation of *d*-3 : 5-diiodotyrosine from the thyroid gland by the action of proteolytic enzymes 1032
HARINGTON, C. R., RANDALL, S. S. and RICHARDSON, G. M. Synthesis of *i*-β-hydroxyglutamic acid. With a note on the dissociation constants 1917
HARRIS, L. J. and INNES, J. R. M. The mode of action of vitamin D. Studies on hypervitaminosis D. The influence of the calcium-phosphate intake 367
HARRISON, D. C. The oxidation of hexose-diphosphoric acid by an enzyme from animal tissues 1011

HARRISON, D. C. Glucose dehydrogenase: a new oxidising enzyme from animal tissues 1016
HARRISON, G. A. Spermine in human tissues 1885
HARRISON, S. T. and MELLANBY, E. A note on the inhibitory effect of monoiodoacetic acid on lactic acid production by cancer tissue 770
HAZLEY, V., *see* BACHARACH, A. L.
Heat-inactivation of proteinase of pancreas (Pace) 1485
Heat-inactivation of trypsin-kinase (Pace) 422
HEILBRON, I. M., GILLAM, A. E. and MORTON, R. A. Specificity in tests for vitamin A. A new conception of the chromogenic constituents of fresh and aged liver oils 1352
HEILBRON, I. M., *see also* GILLAM, A. E. and MORTON, R. A.
HELE, T. S. Studies in the sulphur metabolism of the dog. IX. The synthesis of phenyl sulphate and indoxyl sulphate 1736
HELE, T. S. and PIRIE, N. W. Studies in the sulphur metabolism of the dog. VIII. The metabolism of glutathione compared with that of other cystine derivatives 1095
Hemicelluloses of box-wood (Preece) 1304
Hemicelluloses of the wood of English oak (O'Dwyer) 2017
HEWITT, L. F. Oxidation-reduction potentials of pneumococcus cultures. II. Effect of catalase 169
HEWITT, L. F. Oxidation-reduction potentials of haemolytic streptococci. II. Effect of catalase 858
HEWITT, L. F. Effect of bacteriophage on oxidation-reduction potentials of *B. dysenteriae* (Shiga) cultures 1447
HEWITT, L. F. Effect of lysozyme on the oxidation-reduction potentials of *M. lysodeikticus* cultures 1452
HEWITT, L. F. Effect of bacteriophage on the oxidation-reduction potentials of *B. coli communis* cultures 1641
HEWITT, L. F. Oxidation-reduction potentials of *Staphylococcus* cultures. II. Effect of bacteriophage 2068
Hexosediphosphate, effect of iodoacetic acid on the fermentation of (Macfarlane) 822
Hexosediphosphoric acid, oxidation of, by an enzyme from animal tissues (Harrison) 1011
Hexosemonophosphoric ester, methylation of (King, McLaughlin and Morgan) 310
Hexosemonophosphoric esters (Robison and King) 323
HILDITCH, T. P. and SLEIGHTHOLME, J. J. The glyceride structure of butter-fats 507
HILDITCH, T. P., *see also* BANKS, A. and GILLAM, A. E.
HILL, T. G., *see* HAAS, P.
HIMSWORTH, H. P. The relation of glycosuria to glycaemia and the determination of the renal threshold for glucose 1128
HIMWICH, H. E., FRIEDMAN, H. and SPIERS, M. A. A modification of the method of Stewart and White for the determination of blood-fat, with observations on several species in post-absorptive conditions 1839
HOBSON, R. P. On an enzyme from blow-fly larvae (*Lucilia sericata*) which digests collagen in alkaline solution 1458
HOLMES, B. E., *see* WATCHORN, E.
HOLMES, E. G., *see* ASHFORD, C. A.
HOPKINS, R. H. Selective fermentation. Alcoholic fermentation of mixtures of glucose and fructose by brewer's and Sauterne yeasts 245
HOPKINS, R. H. and KELLY, H. E. The proteolytic enzymes of green malt. II. Action of the proteinase on egg-albumin, caseinogen, edestin and fibrin at different reactions 256
HOPKINS, R. H., KERMACK, W. O. and SPRAGG, W. T. The effect of hydrogen ion concentration on the precipitating and protective actions of proteins on colloidal gold and gum benzoin 829
HOPKINS, R. H., KERMACK, W. O. and SPRAGG, W. T. The effect of hydrogen ion concentration on the precipitating and protective actions of mixtures of two proteins on colloidal gold and gum benzoin 1860
HOPKINS, S. J., *see* PIPER, S. H.
HOWITT, F. O., *see* PRIDEAUX, E. B. R.
HUGGINS, C. B. The phosphatase activity of transplants of the epithelium of the urinary bladder to the abdominal wall producing heterotopic ossification 728
HULME, A. C. and NARAIN, R. The ferricyanide method for the determination of reducing sugars. A modification of the Hagedorn-Jensen-Hanes technique 1051
HUME, E. M. and SMITH, H. H. Calcification of the bones of rats on a diet low in ergosterol 292
HUME, E. M. and SMITH, H. H. The relation of a fat-free diet to the scaly tail condition in rats described by Burr and Burr 300
HUMPHREYS, F. E. and ZILVA, S. S. Metabolism in scurvy. III. The absorption and retention of calcium and phosphorus by guinea-pigs 579
Hydrogenase (Stephenson and Stickland) 205, 215
Hydrogen ion concentration, effect of, on oxidations by *B. coli communis* (Cook and Alcock) 523
Hydrogen ion concentration, effect of, on precipitating and protective actions of proteins on colloidal gold and gum benzoin (Hopkins, Kermack and Spragg) 829, 1860
Hydrogen ion concentration of the potato tuber (Robertson and Smith) 763
i-β-Hydroxyglutamic acid, synthesis and dissociation constants of (Harington, Randall and Richardson) 1917
6-Hydroxy-2-methylbenzoic acid from glucose by the action of *P. griseo-fulvum* (Anslow and Raistrick) 39
Hydroxypyruvic aldehyde (Hynd) 11
HYND, A. Hydroxypyruvic aldehyde: its preparation and physiological behaviour 11
HYND, A. and ROTTER, D. L. Studies on the metabolism of animals on a carbohydrate-free diet. II. Variations in the sensitivity towards insulin of different species of animals on carbohydrate-free diets. 457
HYND, A. and ROTTER, D. L. Studies on the metabolism of animals on a carbohydrate-free diet. III. Effect of alkali on the sensitivity towards insulin of albino rats 1893

Hypervitaminosis D, influence of calcium-phosphate intake on (Harris and Innes) 367

Induction period of zymin, effect of arsenate on (Macfarlane) 822
Innes, J. R. M., *see* Harris, L. J.
Insulin, effect of alkali on sensitivity of albino rats to (Hynd and Rotter) 1893
Insulin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Insulin, effect of, on growth, nitrogen excretion and respiratory metabolism (Goldblatt and Ellis) 221
Insulin, sensitivity to, in relation to cyclic changes in glycogen content of liver and muscles of rats and mice (Ågren, Wilander and Jorpes) 777
Insulin, sensitivity to, of animals on carbohydrate-free diets (Hynd and Rotter) 457
Intestine, surviving, influence of calcium on rate of diffusion of sugars through (Magee and Sen) 643
Invertase, hydrolysis of concentrated sugar solutions by (Auden and Dawson) 1909
Iodine, determination of, in blood (Aitken) 446
Iodoacetate, sodium, effect of, on glyoxalase (Dudley) 439
Iodoacetic acid, effect of, on the fermentation of hexosediphosphate (Macfarlane) 822
Iodoacetic acid, inhibitory effect of, on lactic acid production by cancer tissue (Harrison and Mellanby) 770
Iodobenzene and *p*-iodophenylmercapturic acid, metabolism of, in rodents (Lawrie) 1037
Isoelectric point of proteins, effect of salts on (Przyłęcki) 713; (Przyłęcki and Giedroyć) 1734

Jenkins, S. H. The biological oxidation of carbohydrate solutions. Part I. The oxidation of sucrose and ammonia in sectional percolating filters 147
Jordan-Lloyd, D. The absorption of water by gelatin. VI. The influence of the thickness and original concentration of the gel 1580
Jorpes, E., *see* Ågren, G.
Jowett, M. The phosphatide and cholesterol contents of normal and malignant human tissues 1991
Jowett, M. and Lawson, E. W. Determination of small amounts of phosphatides and cholesterol in tissues 1981

Kelly, H. E., *see* Hopkins, R. H.
Kemp, relation between cystine yield and total sulphur in (Rimington) 71
Kerly, M. The total carbohydrate content of isolated frog muscle 671
Kermack, W. O., *see* Hopkins, R. H.
Ketones, paraffins and secondary alcohols, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
Key, K. M. and Elphick, G. K. A quantitative method for the determination of vitamin C 888
Key, K. M., *see also* Coward, K. H.
Kidd, F., *see* Bracewell, M. F.
Kidney tissue, embryo, growing *in vitro*, effect of fructose, galactose and xylose on ammonia and urea production of (Watchorn and Holmes) 843
King, A. T., *see* Barritt, J. and Bekker, J. G.
King, E. J. The enzymic hydrolysis of lecithin 799
King, E. J., McLaughlin, R. R. and Morgan, W. T. J. The methylation of hexosemonophosphoric ester 310
King, E. J., *see also* Robison, R.
Kodera, S., *see* Sinoda, O.
Kon, S. K. The self-selection of food constituents by the rat 473
Kon, S. K. A study of nitrogen balance in vitamin B_2 deficiency in the rat 482
Kon, S. K. and Markuze, Z. The biological values of the proteins of breads baked from rye and wheat flours alone or combined with yeast or soya bean flour 1476
Kramer, H. and Steiner, A. Notes on the Hagedorn-Jensen method for the determination of blood-sugar 161

Lactic acid content of organs of normal and rice-fed pigeons, effects of rest and exercise on (Fisher) 1410
Lactic acid formation in muscle and amylase, relation between (Case) 561
Lactic acid, oxidation of, in brain tissue (Ashford and Holmes) 2028
Lactic acid production by cancer tissue, inhibitory effect of monoiodoacetic acid on (Harrison and Mellanby) 770
Lamb, J. and Robson, W. The Erlenmeyer synthesis of amino-acids 1231
Laminaria, cell wall of, nature of constituents of (Bird and Haas) 403
Laminaria, mannuronic acid from (Bird and Haas) 403
Lampreys, occurrence of vitamin D in (Callow and Fischmann) 1464
Lawrence, R. D. and McCance, R. A. The effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on the distribution of glycogen in the rat 570
Lawrie, N. R. The metabolism of iodobenzene and *p*-iodophenylmercapturic acid in the rat and the rabbit 1037
Lawson, E. W., *see* Jowett, M.
Leaves, green, lipoid matter extracted from (Collison and Smedley-MacLean) 606
Lecithin, enzymic hydrolysis of (King) 799
Lepper, E. H. and Martin, C. J. On the behaviour of "indifferent" electrodes when used for the determination of oxidation-reduction potentials in the presence of hydrogen 45
Lewis, S. J. The assimilation of aluminium by the human system 2162
Linder, G. C. and Carmichael, E. A. The chlorides and inorganic constituents of the serum and cerebrospinal fluid in nephritis and acidosis 1090
Lipoid matter extracted from green leaves (Collison and Smedley-MacLean) 606
Liver oils, antimony trichloride test and ultra-violet absorption of (Gillam and Morton) 1346

Liver oils, chromogenic constituents of fresh and aged (Heilbron, Gillam and Morton) 1352
Liver oils, spectrographic data concerning (Morton, Heilbron and Thompson) 20
Liver tissue in inanition, glyoxalase-coenzyme ratio of (Vogt-Møller) 1540
Lovern, J. A., Creed, R. H. and Morton, R. A. The Mittelmann process for treating fish-livers 1341
Lovern, J. A. and Morton, R. A. Pigmentation of the liver of the monk (or angler) fish 1336
Lowndes, J., *see* Plimmer, R. H. A.
Lucas, N. S. The permeability of human epidermis to ultra-violet irradiation 57
Lupin seedling, nitrogen metabolism of (McKie) 2181
Lysozyme, effect of, on oxidation-reduction potentials of *M. lysodeikticus* cultures (Hewitt) 1452

McCance, R. A. and Shipp, H. L. The colorimetric determination of sodium 449
McCance, R. A. and Shipp, H. L. The micro-determination of sodium in biological materials 1845
McCance, R. A., *see also* Lawrence, R. D.
McCullagh, D. R. and Case, E. M. A differential resistance thermometer for biological use 1213
McCullagh, D. R. and Case, E. M. Studies on "non-irritable" muscle 1220
Macfarlane, M. G. Fermentation by yeast preparations. I. The effect of monoiodoacetate on the fermentation of hexosediphosphate. II. The action of arsenate on the induction period of zymin 822
Macfarlane, M. G., *see also* Harden, A.
McFarlane, W. D., Graham, W. R., Jr., and Richardson, F. The fat-soluble vitamin requirements of the chick. I. The vitamin A and vitamin D content of fish meal and meat meal 358
McGowan, J. P., Cunningham, I. J., and Auchinachie, D. W. On the fundamental nature of vitamin D action 1295
Mackenzie, K. The biochemistry of aluminium. II. Excretion and absorption of aluminium in the rat 287
McKibbin, I. M. W., *see* Capper, N. S.
McKie, P. The nitrogen metabolism of the lupin seedling 2181
McLaughlin, R. R. The micro-determination of barium 307
McLaughlin, R. R., *see also* King, E. J.
Magee, H. E. and Sen, K. C. The influence of calcium on the rate of diffusion of sugars through surviving intestine 643
Mann, P. J. G. The kinetics of peroxidase action 918
Mannitol, production of, from hexoses and pentoses by white species of *Aspergillus* (Coyne and Raistrick) 1513
Mannuronic acid from *Laminaria* (Bird and Haas) 403
Mapson, L. W., *see* Cook, R. P. and Guha, B. C.
Markuze, Z., *see* Kon, S. K.
Martin, C. J., *see* Lepper, E. H.
Meat meal, vitamin A and vitamin D content of (McFarlane, Graham and Richardson) 358
Meldrum, N. U. The acid-denaturation of haemoglobin 1498
Mellanby, E., *see* Harrison, S. T.
Merulius lachrymans, products of decay of spruce wood rotted by (Barton-Wright and Boswell) 494
Metabolism, calcium and phosphorus of guinea-pigs in scurvy (Humphreys and Zilva) 579
Metabolism, carbohydrate, in birds (Fisher) 1410
Metabolism, carbohydrate, intermediary (Dudley) 439
Metabolism, fat, in muscular exercise (Stewart, Gaddie and Dunlop) 733
Metabolism in tetany (Morris, Watson and Morris) 786
Metabolism, muscle, occurrence of pyruvic acid and methylglyoxal in (Case and Cook) 1319
Metabolism, nitrogen, of seaweeds (Haas and Hill) 1472
Metabolism, nitrogen, of the lupin seedling (McKie) 2181
Metabolism of animals on a carbohydrate-free diet (Hynd and Rotter) 1893
Metabolism of iodobenzene and *p*-iodophenylmercapturic acid in rodents (Lawrie) 1037
Metabolism of normal and tumour tissue (Dickens and Šimer) 973, 985
Metabolism, respiratory, effect of insulin on (Goldblatt and Ellis) 221
Metabolism, sulphur, in the dog (Hele and Pirie) 1095; (Hele) 1736
Methionine, synthesis of (Barger and Weichselbaum) 997
Methylglyoxal, occurrence of, in muscle metabolism (Case and Cook) 1319
Michaelis constant for saccharase, significance of (Briggs) 1801
M. lysodeikticus cultures, effect of lysozyme on oxidation-reduction potentials of (Hewitt) 1452
Micro-organisms, biochemistry of (Coyne and Raistrick) 1513; (Birkinshaw, Charles and Clutterbuck) 1522; (Birkinshaw, Callow and Fischmann) 1977
Milk, influence of sunlight on (Whitehead) 1647
Milk products, alcohol-soluble proteins from (Allen) 1045
Mittelmann process for treating fish-livers (Lovern, Creed and Morton) 1341
Monk (or angler) fish, pigmentation of the liver of (Lovern and Morton) 1336
Moore, T. Vitamin A and carotene. VII. The distribution of vitamin A and carotene in the body of the rat 275
Moore, T. Vitamin A and carotene. VIII. High potency vitamin A concentrates 2131
Moore, T., *see also* Dann, W. J.
Morgan, B. G. E., *see* Coward, K. H.
Morgan, W. T. J., *see* King, E. J.
Morris, N., *see* Morris, S.
Morris, S., Watson, A. McL. and Morris, N. Metabolism studies in tetany 786
Morton, R. A., Heilbron, I. M. and Thompson, A. Spectrographic data concerning vitamin A and liver oils 20

MORTON, R. A., *see also* COWARD, K. H., GILLAM, A. E., HEILBRON, I. M. and LOVERN, J. A.
Muscle, frog, isolated, carbohydrate content of (Kerly) 671
Muscle metabolism, occurrence of pyruvic acid and methylglyoxal in (Case and Cook) 1319
Muscle, "non-irritable" (McCullagh and Case) 1220

NARAIN, R., *see* HULME, A. C.
NARAYANA, B., *see* REID, C.
NARAYANAMURTI, D. and RAMASWAMI, C. V. The nature of tyrosinase 749
Narcotics, effect of, on dehydrogenases (Sen) 849
Nematospora gossypii, accessory factor necessary for the growth of (Buston and Pramanik) 1656, 1671
Nephritis, chlorides and inorganic constituents of serum and cerebrospinal fluid in (Linder and Carmichael) 1090
NICHOLSON, W. N., NIERENSTEIN, M., POOL, J. C. and PRICE, N. V. The action of tannase on gallotannin 752
NIERENSTEIN, M., *see* NICHOLSON, W. N.
NILSSON, R., *see* v. EULER, H.
Nitrates, reduction of, by *B. coli* (Stickland) 1543
Nitrogen balance in vitamin B_2 deficiency in the rat (Kon) 482
Nitrogen, dicarboxylic acid-, of proteins (Damodaran) 2123
Nitrogen, distribution of, in urine during increased catabolism (Cuthbertson) 236
Nitrogen excretion, effect of insulin on (Goldblatt and Ellis) 221
Nitrogen immobilisation during decomposition of plant materials (Richards and Norman) 1769
Nitrogen immobilisation by straw, effect of p_H on (Norman) 1779
Nitrogen, liberation of elementary, by bacteria (Barritt) 1965
Nitrogen metabolism of the lupin seedling (McKie) 2181
Nitrogen, urinary output of, in relation to cyclic changes in glycogen content of liver and muscles of rats and mice (Ågren, Wilander and Jorpes) 777
"Non-irritable" muscle (McCullagh and Case) 1220
NORMAN, A. G. Studies on the gums. II. Tragacanthin—the soluble constituent of gum tragacanth 200
NORMAN, A. G. The biological decomposition of plant materials. VI. The effect of hydrogen ion concentration on the rate of immobilisation of nitrogen by straw 1779
NORMAN, A. G., *see also* RICHARDS, E. H.
Nutrition, copper in animal (Cunningham) 1267
Nutrition, experiments on (Plimmer, Raymond and Lowndes) 1788

Obituary notice of James Lorrain Smith 1849
O'DWYER, M. H. The hemicelluloses of the wood of English oak. I. The effect of the drying of the wood on the yields and composition of hemicellulose A 2017
Oil development in the seed of a growing plant (Eyre) 1902
Oleic acid, oxidation of, by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Orange, vitamin C in (Bracewell and Zilva) 1081
OSBORN, T. W. B. A study of the factors influencing the concentration of complement in the blood 2136
Ossification, heterotopic, produced by phosphatase activity of transplants of epithelium of urinary bladder (Huggins) 728
Ovalbumin and gelatin, electrophoretic velocities of, in their mixtures (Prideaux and Howitt) 391
Ovary, fowl's, growth and chemical composition of (Romanoff) 994
OVERHOFF, J., *see* BRUINS, H. R.
Oxidation by *B. coli communis*, effect of hydrogen ion concentration on (Cook and Alcock) 523
Oxidation of oleic acid by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Oxidation of sulphydryl compounds by hydrogen peroxide (Pirie) 1565
Oxidation-reduction potentials, behaviour of indifferent electrodes in determination of, in presence of hydrogen (Lepper and Martin) 45
Oxidation-reduction potentials of *B. coli communis* cultures, effect of bacteriophage on (Hewitt) 1641
Oxidation-reduction potentials of *B. dysenteriae* cultures, effect of bacteriophage on (Hewitt) 1447
Oxidation-reduction potentials of haemolytic streptococci, effect of catalase on (Hewitt) 858
Oxidation-reduction potentials of *M. lysodeikticus* cultures, effect of lysozyme on (Hewitt) 1452
Oxidation-reduction potentials of pneumo coccus cultures, effect of catalase on (Hewitt) 169
Oxidation-reduction potentials of *Staphylococcus* cultures, effect of bacteriophage on (Hewitt) 2068
Oxidations, biological, in succinic acid series (Quastel and Wheatley) 117
Oxidations, effect of dyestuffs on (Quastel and Wheatley) 629
Oxido-reductions, activation of, by co-zymase (v. Euler and Nilsson) 2168
Oxycoccus macrocarpus Pers., anthocyanin of (Grove and Robinson) 1706
OYA, C., *see* SINODA, O.

PACE, J. The effect of heat upon the activating efficiency of enterokinase 1
PACE, J. The inactivation of trypsin-kinase by heat and the effect of added protein thereon 422
PACE, J. The heat-inactivation of the proteinase of the pancreas 1485
Paraffins, ketones and secondary alcohols, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
PATTERSON, L. M. B. The effects of cyanide and some other salts on fermentation by yeast preparations 1593

PEARCE, M. S. B., *see* SMEDLEY-MACLEAN, I.
Peas, loss of glucose from dried, on soaking (Clifford) 1999
Penicillium griseo-fulvum, production of hydroxymethylbenzoic acid from glucose by action of (Anslow and Raistrick) 39
Penicillium puberulum Bainier, isolation of ergosterol from (Birkinshaw, Callow and Fischmann) 1977
Pepsin, enzymic synthesis of proteins by (Cuthbertson and Tompsett) 2004
Peroxidase action, kinetics of (Mann) 918
PETERS, R. A., *see* GAVRILESCU, N.
Phloridzin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Phosphatase activity of transplants of epithelium of urinary bladder (Huggins) 728
Phosphatases, blood- (Roche) 1724
Phosphate buffer mixtures, vapour pressure depressions of, at 20·3° (Grollman) 166
Phosphatide and cholesterol contents of normal and malignant human tissues (Jowett) 1991
Phosphatides and cholesterol, determination of small amounts of (Jowett and Lawson) 1981
Phosphorus metabolism of guinea-pigs in scurvy (Humphreys and Zilva) 579
Pigmentation of the liver of the monk (or angler) fish (Lovern and Morton) 1336
PIPER, S. H., CHIBNALL, A. C., HOPKINS, S. J., POLLARD, A., SMITH, J. A. B. and WILLIAMS, E. F. Synthesis and crystal spacings of certain long-chain paraffins, ketones and secondary alcohols 2072
PIPER, S. H., *see also* CHIBNALL, A. C. and POLLARD, A.
PIRIE, N. W. The cuprous derivatives of some sulphydryl compounds 614
PIRIE, N. W. The oxidation of sulphydryl compounds by hydrogen peroxide. I. Catalysis of oxidation of cysteine and glutathione by iron and copper 1565
PIRIE, N. W., *see also* HELE, T. S.
Pituitrin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Plant materials, biological decomposition of (Richards and Norman) 1769, (Norman) 1779
Plant, oil development in seed of growing (Eyre) 1902
Plastein formation (Cuthbertson and Tompsett) 2004
PLEASS, W. B. The absorption of water by gelatin. VII. The influence of temperature on swelling in acid solutions and the combination of gelatin with hydrochloric, nitric or sulphuric acid 1943
PLIMMER, R. H. A., RAYMOND, W. H. and LOWNDES, J. Experiments on nutrition. X. Comparative vitamin B_1 values of foodstuffs. Cereals II 691
PLIMMER, R. H. A., RAYMOND, W. H. and LOWNDES, J. Experiments on nutrition. XI. Comparative vitamin B_1 values of foodstuffs. Fruits and vegetables 1788
Pneumococcus cultures, oxidation-reduction potentials of, effect of catalase on (Hewitt) 169
POLLARD, A., CHIBNALL, A. C. and PIPER, S. H. The wax constituents of forage grasses. I. Cocksfoot and perennial ryegrass 2111
POLLARD, A., *see also* CHIBNALL, A. C. and PIPER, S. H.
POOL, J. C., *see* NICHOLSON, W. N.
POPE, C. G. A method for determining aluminium in certain aluminium protein compounds 1949
Potato tuber, hydrogen ion concentration of (Robertson and Smith) 763
Potatoes, blackening of, after cooking (Tinkler) 773
PRAMANIK, B. N., *see* BUSTON, H. W.
PREECE, I. A. Studies on hemicelluloses. IV. The proximate analysis of box-wood, and the nature of its furfuraldehyde-yielding constituents 1304
PRENTICE, J. H., *see* CAPPER, N. S.
PRICE, H. I. Autolysis in malignant and normal rabbit tissues 1491
PRICE, N. V., *see* NICHOLSON, W. N.
PRIDEAUX, E. B. R. and HOWITT, F. O. The electrophoretic velocities of gelatin and ovalbumin in different concentrations of their mixtures and the effect of ultra-violet irradiation 391
Proteases, bacterial, formation of, in synthetic media (Haines) 1851
Protein, alcohol-soluble from ragi (*Eleusine coracana*) (Damodaran) 2123
Proteinase of green malt, effect of, on egg-albumin, caseinogen, edestin and fibrin (Hopkins and Kelly) 256
Proteinase of the pancreas, heat-inactivation of (Pace) 1485
Proteins, alcohol-soluble, from milk products (Allen) 1045
Proteins and protein derivatives, influence of, on enzymic hydrolysis of starch by malt diastase (Filipowicz) 1874
Proteins, determination of cystine in (Tompsett) 2014
Proteins, dicarboxylic acid-nitrogen of (Damodaran) 2123
Proteins, effect of p_H on precipitating and protective action of mixtures of, on colloidal gold and gum benzoin (Hopkins, Kermack and Spragg) 829, 1860
Proteins, effect of salts on isoelectric point of (Przyłęcki) 713; (Przyłęcki and Giedroyć) 1734
Proteins, enzymic synthesis of (Cuthbertson and Tompsett) 2004
Proteins of breads, biological values of (Kon and Markuze) 1476
Proteins, precipitating and protective actions of, on colloidal gold and gum benzoin, effect of hydrogen ion concentration on (Hopkins, Kermack and Spragg) 829, 1860
Proteins, serum, carbohydrate complex of (Rimington) 1062
Proteolytic enzymes of green malt (Hopkins and Kelly) 256
PRZYŁĘCKI, S. J. Effect of addition of salts on the isoelectric point of proteins 713
PRZYŁĘCKI, S. J. and GIEDROYĆ, W. Effect of addition of salts on the isoelectric point of proteins II. 1734
PRZYŁĘCKI, S. J., *see also* GIEDROYĆ, G.

Pyruvic acid, occurrence of, in muscle metabolism (Case and Cook) 1319

QUASTEL, J. H. The action of dyestuffs on enzymes. II. Fumarase 898
QUASTEL, J. H. Trypanocidal action and toxicity to enzymes 1121
QUASTEL, J. H. and WHEATLEY, A. H. M. Biological oxidations in the succinic acid series 117
QUASTEL, J. H. and WHEATLEY, A. H. M. The action of dyestuffs on enzymes. I. Dyestuffs and oxidations 629
Quills, porcupine, sulphur distribution in component structures of (Bekker and King) 1077

Radiation, ultra-violet, effect of, on electrophoretic velocities of gelatin and ovalbumin in their mixtures (Prideaux and Howitt) 391
Radiation, ultra-violet, permeability of human epidermis to (Lucas) 57
RAISTRICK, H., *see* COYNE, F. P. and ANSLOW, W. K.
RAMAGE, H., *see* SHELDON, J. H.
RAMASWAMI, C. V., *see* NARAYANAMURTI, D.
RANDALL, S. S., *see* HARINGTON, C. R.
Rat, normal nutrition of, factors necessary for (Guha) 960
Rat, self-selection of food constituents by the (Kon) 473
RAYMOND, W. H., *see* PLIMMER, R. H. A.
Reductase, reactivation of, in washed yeast preparations (Harden and Macfarlane) 818
REERINK, E. H. and VAN WIJK, A. The vitamin D problem. II. Optical rotation of vitamin D 1001
REEVES, H. G. and RENBOM, E. T. Note on dihydroxyacetone 412
REID, C. and NARAYANA, B. Studies in blood glycolysis. Preliminary observations 339
Renal threshold for glucose, determination of (Himsworth) 1128
RENBOM, E. T., *see* REEVES, H. G.
Respiratory catalysts of *B. coli*, relationship between (Cook, Haldane and Mapson) 534
Respiratory quotient of tissues, measurement of (Dickens and Šimer) 973, 985
"Resting" bacteria (Sandiford and Wooldridge) 2172
RICHARDS, E. H. and NORMAN, A. G. The biological decomposition of plant materials. V. Some factors determining the quantity of nitrogen immobilised during decomposition 1769
RICHARDSON, F., *see* MCFARLANE, W. D.
RICHARDSON, G. M., *see* HARINGTON, C. R.
RIMINGTON, C. The relation between cystine yield and total sulphur in kemp and outercoat animal fibres 71
RIMINGTON, C. The carbohydrate complex of the serum-proteins. II. Improved method for isolation and re-determination of structure. Isolation of glucosaminodimannose from proteins of ox blood 1062
RIMINGTON, C., *see also* BARRITT, J. and STEWART, A. M.
ROBERTSON, I. M. and SMITH, A. M. A study of the hydrogen ion concentration of the potato tuber 763
ROBINSON, G. M. and ROBINSON, R. A survey of anthocyanins. I 1687
ROBINSON, R., *see* GROVE, K. E. and ROBINSON, G. M.
ROBISON, R. and KING, E. J. Hexosemonophosphoric esters 323
ROBSON, W., *see* LAMB, J.
ROCHE, J. Blood-phosphatases 1724
ROMANOFF, A. L. Growth and chemical composition of functioning fowl's ovary (*Gallus domesticus*) 994
ROSCOE, M. H. The distribution of the vitamin B complex. II. Root vegetables 1205
ROSCOE, M. H. The distribution of the vitamin B complex. III. Fruits 2050
ROSCOE, M. H. The effects of coprophagy in rats deprived of the vitamin B complex 2056
ROSENHEIM, O. and CALLOW, R. K. Colour reactions of sterols with nitric acid 74
ROTTER, D. L., *see* HYND, A.

Saccharase, significance of Michaelis constant for (Briggs) 1801
SALT, H. B. The colorimetric determination of uric acid in urine 1720
SALT, H. B. The ergothioneine content of the blood in health and disease 1712
Salts, effect of addition of, on the p_H of solutions of ampholytes (Giedroyć and Przyłęcki) 465
Salts, effect of, on isoelectric point of proteins (Przyłęcki) 713
SANDIFORD, B. R. and WOOLDRIDGE, W. R. "Resting" bacteria 2172
Scaly tail condition in rats, relation of fat-free diet to (Hume and Smith) 300
SCHIØDT, E. On the quantity of free water in the red blood corpuscles 8
SCHULTZER, P. Investigations on the determination of vitamin D. Comparison between the preventive and the curative methods 1745
Scurvy, calcium and phosphorus metabolism of guinea-pigs in (Humphreys and Zilva) 579
SELBY, D. L., *see* HARDING, V. J.
SEN, K. C. The effect of narcotics on some dehydrogenases 849
SEN, K. C. The sensitisation of red blood corpuscles in haemolysis 1244
SEN, K. C., *see also* MAGEE, H. E.
Serum, action of, on trypsin (Fine) 647
Serum, chlorides and inorganic constituents of, in nephritis and acidosis (Linder and Carmichael) 1090
SHELDON, J. H. and RAMAGE, H. A spectrographic analysis of human tissues 1608
SHIPP, H. L., *see* MCCANCE, R. A.
DA SILVA, G. A. The determination of tryptophan and tyrosine 1634
ŠIMER, F., *see* DICKENS, F.
SINODA, O., KODERA, S. and OYA, C. The chemistry of cooking. I. The chemical changes of carbohydrates in the sweet potato according to various methods of cooking 1973
SLEIGHTHOLME, J. J., *see* HILDITCH, T. P.

SMEDLEY-MACLEAN, I. and PEARCE, M. S. B. The oxidation of oleic acid by means of hydrogen peroxide with and without the addition of copper sulphate: a possible analogy with its oxidation *in vivo* 1252
SMEDLEY-MACLEAN, I., *see also* COLLISON, D. L.
SMITH, A. M., *see* ROBERTSON, I. M.
SMITH, H. H., *see* HUME, E. M.
SMITH, J. A. B., *see* CHIBNALL, A. C. and PIPER, S. H.
Sodium, determination of, colorimetrically (McCance and Shipp) 449
Sodium, micro-determination of, in biological materials (McCance and Shipp) 1845
Softwood, drying of a (Campbell and Booth) 756
Spectrographic analysis of human tissues (Sheldon and Ramage) 1608
Spermine in human tissues (Harrison) 1885
SPIERS, M. A., *see* HIMWICH, H. E.
Spinach, lipoid matter extracted from (Collison and Smedley-MacLean) 606
SPRAGG, W. T., *see* HOPKINS, R. H.
Spruce wood, products of decay of, rotted by *Merulius lachrymans* (Barton-Wright and Boswell) 494
Staphylococcus cultures, oxidation-reduction potentials of, effect of bacteriophage on (Hewitt) 2068
Starch, estimation of products of hydrolysis of, by taka-diastase (Widdowson) 863
Starvation, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
STEDMAN, E. and STEDMAN, E. Studies on the relationship between chemical constitution and physiological action. III. The inhibitory action of certain synthetic urethanes on the activity of liver esterase 1147
STEINER, A., *see* KRAMER, H.
STENT, H. B., *see* WALKER, T. K.
STEPHENSON, M. and STICKLAND, L. H. Hydrogenase, a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme 205
STEPHENSON, M. and STICKLAND, L. H. Hydrogenase. II. The reduction of sulphate to sulphide by molecular hydrogen 215
Sterols, colour reactions of, with nitric acid (Rosenheim and Callow) 74
STEWART, A. M. and RIMINGTON, C. The basic amino-acids of wool 2189
STEWART, C. P., GADDIE, R. and DUNLOP, D. M. Fat metabolism in muscular exercise 733
STICKLAND, L. H. The reduction of nitrates by *B. coli* 1543
STICKLAND, L. H., *see also* STEPHENSON, M.
Stout as an addition to white wheat flour or fish meal as a diet for pigs (Blissett and Golding) 349
Streptococci, haemolytic, effect of catalase on oxidation-reduction potentials of (Hewitt) 858
SUBRAMANIAM, V., *see* WALKER, T. K.
Succinic acid series, biological oxidations in (Quastel and Wheatley) 117
Sucrose, oxidation of, in percolating filters (Jenkins) 147
Sucrose solutions, dilute, biological filtration of (Barritt) 1419
Sugar alcohols, occurrence of, in marine algae (Haas and Hill) 1470
Sugar, fermentable, in normal urine (Harding and Selby) 1815
Sugar solutions, hydrolysis of concentrated, by invertase (Auden and Dawson) 1909
Sugars, reducing, determination of, iodimetrically, in the apple (Archbold and Widdowson) 101
Sugars, reducing, ferricyanide method for determination of (Hulme and Narain) 1051
Sugars, reducing, mixed, determination of small quantities of (Widdowson) 863
Sulphate, enzymic reduction of, by molecular hydrogen (Stephenson and Stickland) 215
Sulphate, inorganic, content and determination of, in the blood (Cuthbertson and Tompsett) 1237
Sulphate, inorganic, determination of, in human blood-plasma (Cope) 1183
Sulphates, ethereal, synthesis in the dog (Hele) 1736
Sulphur, distribution of, in urine during increased catabolism (Cuthbertson) 236
Sulphur metabolism of the dog (Hele) 1736
Sulphur metabolism of the dog (Hele and Pirie) 1095
Sulphydryl compounds, cuprous derivatives of (Pirie) 614
Sulphydryl compounds, oxidation of, by hydrogen peroxide (Pirie) 1565
Sunlight, influence of, on milk (Whitehead) 1647

Taka-diastase, hydrolysis of starch by (Widdowson) 863
Tallows, beef, glyceride structure of (Banks and Hilditch) 1168
Tannase, action of, on gallotannin (Nicholson, Nierenstein, Pool and Price) 752
Tetany, metabolism in (Morris, Watson and Morris) 786
Thermometer, a differential resistance, for biological use (McCullagh and Case) 1213
THOMPSON, A., *see* MORTON, R. A.
Thyroid, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Thyroid gland, isolation of *d*-3 : 5-diiodotyrosine from (Harington and Randall) 1032
Thyroxine, effect of arginine on body weight of tumour-bearing mice injected with (Gilroy) 414
Timber, chemical aspect of drying of (Campbell and Booth) 756
TINKLER, C. K. The blackening of potatoes after cooking 773
Tissues, human, spectrographic analysis of (Sheldon and Ramage) 1608
Tissues, human, spermine in (Harrison) 1885
Tissues, normal and malignant human, phosphatide and cholesterol contents of (Jowett) 1991
Tissues, rabbit, autolysis in malignant and normal (Price) 1491
TOMPSETT, S. L. A note on the determination of cystine in proteins by the method of Folin and Marenzi 2014

TOMPSETT, S. L., *see also* CUTHBERTSON, D. P.
Torulin, function of. An *in vitro* effect (Gavrilescu and Peters) 2150
Tragacanthin (Norman) 200
Trypanocidal action and toxicity to enzymes (Quastel) 1121
Trypsin, action of serum on (Fine) 647
Trypsin-kinase, inactivation of, by heat (Pace) 422
Tryptophan, determination of (da Silva) 1634
Tumour-bearing mice injected with thyroxine, effect of arginine on the body weight of (Gilroy) 414
Tumour tissue, metabolism of (Dickens and Šimer) 973, 985
TURNER, K. The fatty acids of the cat's kidney. I 49
Tyrosinase (Narayanamurti and Ramaswami) 749
Tyrosine, determination of (da Silva) 1634

Ultra-violet absorption of liver oils (Gillam and Morton) 1346
Urea in urine, method for direct determination of (Cole) 1653
Urease-urea-gelatin system (Freiberger) 705
Urethanes, inhibitory action of, on activity of liver esterase (Stedman and Stedman) 1147
Uric acid, colorimetric determination of, in urine (Salt) 1720
Urine, "ether reaction" of (Boyd) 812
Urine, normal, fermentable sugar in (Harding and Selby) 1815

VAHLQUIST, B. Blood-phosphorus in fat absorption 1628
Vegetables, root, distribution of vitamin B complex in (Roscoe) 1205
Vitamin A and carotene (Moore) 2131
Vitamin A content of fish meal and meat meal (McFarlane, Graham and Richardson) 358
Vitamin A, conversion of carotene into, by fowl (Capper, McKibbin and Prentice) 265
Vitamin A, determination of, in cod-liver oils (Coward, Dyer, Morton and Gaddum) 1102
Vitamin A, distribution of, in the body of the rat (Moore) 275
Vitamin A, effect of addition of, to a rachitogenic diet (Bacharach, Allchorne and Hazley) 639
Vitamin A, molecular weight of (Bruins, Overhoff and Wolff) 430
Vitamin A reserves of the rat, effect on, of vitamin B deficiency (Dann and Moore) 914
Vitamin A, specificity in tests for (Heilbron, Gillam and Morton) 1352
Vitamin A, spectrographic data concerning (Morton, Heilbron and Thompson) 20
Vitamin A, spectrographic data of natural fats and their fatty acids in relation to (Gillam, Heilbron, Hilditch and Morton) 30
Vitamin B complex, distribution of (Roscoe) 1205
Vitamin B complex, distribution of, in fruits (Roscoe) 2050
Vitamin B complex, effects of coprophagy in rats deprived of (Roscoe) 2056
Vitamin B deficiency, biochemical lesions in (Gavrilescu and Peters) 1397
Vitamin B deficiency, effect of, on vitamin A reserves of the rat (Dann and Moore) 914
Vitamin B_1, chemical nature of, from evidence afforded by its electrical transference (Birch and Guha) 1391
Vitamin B_1 concentrates from yeast, preparation and behaviour of (Guha) 931
Vitamin B_1 deficiency, blood-sugar in (Bell) 1755
Vitamin B_1 physiological function of (Guha) 1367
Vitamin B_1 values of cereals (Plimmer, Raymond and Lowndes) 691
Vitamin B_1 values of fruits and vegetables (Plimmer, Raymond and Lowndes) 1788
Vitamin B_2 deficiency in the rat, nitrogen balance in (Kon) 482
Vitamin B_2, sources, stability and chemistry of (Guha) 945
Vitamin C, estimation of (Key and Elphick) 888
Vitamin C in orange and grape fruit (Bracewell and Zilva) 1081
Vitamin concentrates, an *in vitro* effect of antineuritic (Gavrilescu and Peters) 2150
Vitamin D content of fish meal and meat meal (McFarlane, Graham and Richardson) 358
Vitamin D, determination of (Schultzer) 1745
Vitamin D, fundamental nature of action of (McGowan, Cunningham and Auchinachie) 1295
Vitamin D, mode of action of (Harris and Innes) 367
Vitamin D, occurrence of, in lampreys (Callow and Fischmann) 1464
Vitamin D, optical rotation of (Reerink and van Wijk) 1001
Vitamin tests, caseinogen for (Coward, Key, Dyer and Morgan) 551
VOGT-MØLLER, P. Is avitaminosis B_1 an intoxication by methylglyoxal? Glyoxalase-co-enzyme ratio in experimental beriberi 418
VOGT-MØLLER, P. On the glyoxalase-co-enzyme ratio in liver tissue in inanition 1540

WALKER, T., SUBRAMANIAM, V., STENT, H. B. and BUTTERWORTH, J. A study of the mechanism of the degradation of citric acid by *B. pyocyaneus* (*Pseudomonas pyocyanea*). II. Action of *B. pyocyaneus* on succinic acid 129
WALLACE, T., *see* BRACEWELL, M. F.
WATCHORN, E. and HOLMES, B. E. Studies in the metabolism of tissues growing *in vitro*. IV. Effect of fructose, galactose and xylose upon the ammonia and urea production of embryo kidney tissue 843
WATSON, A. McL., *see* MORRIS, S.
Wax constituents of apple cuticle (Chibnall, Piper, Pollard, Smith and Williams) 2095
Wax constituents of forage grasses (Pollard, Chibnall and Piper) 2111
WEICHSELBAUM, T. E., *see* BARGER, G.
WEST, C., *see* BRACEWELL, M. F.

Wheat flour, white, inadequacy of, for prolonged growth of pigs (Blissett and Golding) 349
Wheatley, A. H. M., *see* Quastel, J. H.
White rots of wood, chemistry of, II (Campbell) 2023
Whitehead, H. R. The influence of sunlight on milk 1647
Widdowson, E. M. A method for the determination of small quantities of mixed reducing sugars and its application to the estimation of the products of hydrolysis of starch by taka-diastase 863
Widdowson, E. M., *see also* Archbold, H. K.
Van Wijk, A., *see* Reerink, E. H.
Wilander, O., *see* Ågren, G.
Williams, E. F., *see* Chibnall, A. C. and Piper, S. H.
Wolff, L. K., *see* Bruins, H. R.
Wood, chemistry of white rots of (Campbell) 2023
Wood of English oak, hemicelluloses of (O'Dwyer) 2017
Wool and porcupine quills, sulphur distribution in component structures of (Bekker and King) 1077
Wool, basic amino-acids of (Stewart and Rimington) 2189
Wool, black-face, sulphur-free nature of medulla in (Barritt and King) 1075
Wooldridge, W. R., *see* Sandiford, B. R.
Woolf, B. The addition compound theory of enzyme action 342

Xylose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843

Yeast, as an addition to white wheat flour or fish meal as a diet for pigs (Blissett and Golding) 349
Yeast, preparation and behaviour of vitamin B_1 concentrates from (Guha) 931
Yeast preparations, effects of cyanide and other salts on fermentation by (Patterson) 1593
Yeasts, brewer's and Sauterne, selective fermentation of glucose and fructose by (Hopkins) 245

Zilva, S. S. A note on the reprecipitation of the antiscorbutic factor from decitrated lemon juice 594
Zilva, S. S., *see also* Bracewell, M. F. and Humphreys, F. E.
Zymin, induction period of, effect of arsenate on (Macfarlane) 822

CAMBRIDGE: PRINTED BY W. LEWIS, M.A., AT THE UNIVERSITY PRESS